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IONIZING RADIATION AND THE CELL

BY

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Editor

Roy Waldo Miner

IONIZING RADIATION AND THE CELL*

Conference Chairman and Consulting Editor

Leslie F. Nims

CONTENTS

Ionizing Radiation Methods for Determining Biological Structure. *By* Ernest Pollard 469

Radiation Studies of Proteins and Enzymes. *By* Richard Setlow 471

Bacterial Virus: Radiation and Function. *By* D. J. Fluke and E. C. Pollard 484

Use of Charged Particles to Measure Skin Thickness and Other Surface Properties. *By* Franklin Hutchinson 494

Irradiation of Parts of Individual Cells. III. Effects of Chromosomal and Extrachromosomal Irradiation on Chromosome Movements. *By* William Bloom, Raymond E. Zirkle, and Robert B. Uretz 503

The Effects of X Rays, 2-MEV Electrons, Thermal Neutrons, and Fast Neutrons on Dormant Seeds of Barley. *By* Richard S. Caldecott 514

Comparative Studies of X Ray-Induced Forward and Reverse Mutation. *By* Norman H. Giles, Frederick J. de Serres, and C. W. H. Partridge 536

Cytochemical Studies of Changes Induced in Cellular Materials by Ionizing Radiations. *By* Berwind P. Kaufmann, Margaret R. McDonald and Maurice H. Bernstein 553

Track Effect in Iochemistry of Aqueous Solutions. *By* Hugo Fricke 567

The Effect of Ionizing Radiations on Systems of Biological Importance. *By* E. S. Guzman Barron 574

Studies on Factors Affecting the Sensitivity of Bacteria to High Velocity Electrons. *By* W. D. Bellamy and E. J. Lawton 595

The Influence of Pretreatments and Posttreatments on Bacterial Inactivation by Ionizing Radiations. *By* George E. Stapleton 604

Radiation Effects in *Paramecium*. *By* E. L. Powers 619

The Role of Oxygen and Peroxide in the Production of Radiation Damage in *Paramecium*. *By* R. F. Kimball 638

Factors in the Radiosensitivity of Mammalian Cells. *By* Harvey M. Patt 649

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IONIZING RADIATION METHODS FOR DETERMINING BIOLOGICAL STRUCTURE

By Ernest Pollard
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Ionizing radiation has played an important part in the past in determining biological structure. In the quite early days, it played an important part in determining the reality of the association between chromosomes and linkage groups in Mendelian genetics. It played an important part in determining the nature of the Kappa factor in *Paramecium*. It cannot, however, be used casually and easily because there are attendant difficulties in interpretation of radiation results.

A large part of the work in the Biophysics Department at Yale has been concerned with an attempt to find the conditions under which the action of radiation can be employed for determining biological structure. Some progress has been made along these lines and it is now possible to indicate what can be done and what results could be yielded by the method.

The major factor in using ionizing radiation to determine structure is the preservation of the space relations of the energy releases which take place when a system is bombarded. This factor makes it essential to avoid irradiating either water or gas in which the energy originally deposited can move around by diffusion. Therefore, either dry or highly viscous preparations have to be irradiated. At first sight, this is a grave limitation but is no more grave than is faced by electron microscopy. Irradiation methods, moreover, have the advantage of the electron microscope, in that there is a relationship between function and what is observed. It is the exploitation of this loss of function due to ionizing radiation which is the power of the method of studying structure in this way.

In order to make the background of radiation action more suitable for its use as a tool in structure study, a wide class of biological molecules has been irradiated covering many enzymes, nucleic acids, and polysaccharides. The result found is that the release of ionization within these molecules in general removes their biological function. It is not always sufficient to release one ion pair within a molecule to achieve this result. It is found that up to four or even five ionizations may be necessary for the removal of certain functions, and one very important feature of the use of ionizing radiation for structural studies turns on the ability to measure this limiting energy. If only one source of radiation is employed, this amount remains an unknown quantity and the interpretation of the findings is ambiguous. If relatively accurate data can be taken with a variety of radiation sources, then it becomes possible to determine the sensitivity of the molecular structure involved and, from this data, to determine something about its size and shape, and its relation to function.

The approach which is used, therefore, is the following. A system, such as a virus, bacterial spore, or a mitochondrial system, is irradiated with fast electrons, with slow, medium, and fast deuterons, and with alpha particles of two velocities. The loss of the various properties in these systems are then studied under all these conditions. In the case of a virus, for example, the ability to infect is studied, as one of the properties. Others will involve the ability to interfere, the ability to attach, the length of the latent period, the combination with antiserum, and so on. The numerical relationship for these inactivations are different in each case. For serological properties, the cross section (which is a parameter representing the probability of inactivation) is generally small. For infectivity, it is generally large. By running the whole gamut of these properties and by then putting together an inferential model of the system, it is possible to make a first attempt at formulating a structure for the particular system involved. For each of these properties, there will be found not only a maximum cross section corresponding to the effect of densely ionizing radiation upon the system, but a variation of the apparent cross section with the bombarding energy of the particle. This variation may either be very simple, corresponding to a very sensitive molecule, or it may be strongly sigmoid in character. From the nature of this curve, it is possible to determine the critical energy which is required for inactivation. Once this is done, the values for inactivation by heavily ionizing particles and by fast electrons permit a determination of the volume, area, and thickness for the particular system which is being studied.

One example of this type of study is given elsewhere in this monograph by Fluke and the author.

In addition to this type of study, a very powerful method has been introduced by Hutchinson. This method employs penetration-limited particles as a method of probing structure. It is sometimes possible to confirm the results of bombardment by the method just described by penetration studies. If these two agree, then the inferred structure starts to have some reasonable validity. If, in addition, the inferred structure from radiation work fits in well with inferences from other methods of study, then the two can be thought of as supplementing one another.

It should be emphasized that radiation alone is not the best method of inferring a structure for a submicroscopic particle. It is wise to confirm this by other methods, as by X-ray diffraction, ultraviolet absorption, and so on. Radiation, therefore, takes its place alongside other methods producing inferred structures, and can be helpful in that regard.

RADIATION STUDIES OF PROTEINS AND ENZYMES*

By Richard Setlow

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Introduction

There is a good deal of interest at present in the action of radiation on living systems and their components. The relationship between the inactivation dose for viruses, for example, and the type of radiation used can give information about the internal structure of virus particles.[1] This phase of radiation action will be discussed in other parts of this symposium. Before we analyze with any degree of certainty the results of radiation action on viruses, we must know what radiation's effects are on still smaller structures such as protein molecules. This is not the only reason to study the action of radiation on proteins, because this action is of interest in its own right and, as has been shown by Pollard,[2] may be used to determine the molecular weights of enzymes.

We shall be concerned in what follows with the *direct* action of radiation on proteins and enzymes. The irradiations to be discussed have all been carried out on dry materials. We do not have to worry about the inactivation of enzymes by free radicals in the surrounding medium. We shall be concerned primarily with the effects of radiation in conjunction with various physical conditions of protein molecules during and after irradiation, and shall make no attempt to give a detailed physical picture of the atomic and molecular processes which give rise to these effects.

Theory and Simple Examples

Ionizing radiation may be used as a tool to investigate molecular structure, because the energy liberated by fast charged particles occurs randomly, but in an average fashion which is well known from physical theory. Most of the energy is lost in the form of primary ionizations, each of which represents, on the average, the loss of 110 e.v. by the incident particle (see Pollard[2] for a review of this subject). It has been shown that the inactivation of an enzyme by incident charged particles may be represented by the equation

$$n/n_0 = e^{-SD} \tag{1}$$

in which n/n_0 represents the relative activity after a dose of radiation D particles per cm.[2] S is known as the inactivation cross section of the molecule, and it represents the chance that an incident particle will inactivate it. It has the units of area per incident particle. The study of the direct action of radiation on proteins and enzymes is the determination of the cross section for different types of incident particles and different conditions during and after irradiation. Because the energy releases of a fast charged particle are sometimes separated by distances which are large compared to the depth of a molecule, every particle passing through a molecule cannot possibly inactivate it, because energy is not always released in the molecule. If we let t represent

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* The work reported in this paper was assisted by the United States Atomic Energy Commission.

the molecular thickness, and i the number of primary ionizations per centimeter of path of the incident particle, then the inactivation cross section is related to the cross section for infinite rate of energy loss, S_0, by the equation

$$S = S_0(1 - e^{-it}) \tag{2}$$

If, as is the case for high energy electrons, the ionization is very sparse, EQUATION 2 reduces to

$$S = (S_0 t)\cdot i = V\cdot i \tag{3}$$

and EQUATION 1 reduces to

$$n/n_0 = e^{-VI} \tag{4}$$

I is the number of primary ionizations per unit volume, and V is the sensitive volume of the molecule. By using a variety of incident particles ranging from electrons (energy loss 2.5 e.v./100 Å.) to alpha particles (energy loss 1400 e.v./100 Å.) it is possible to determine the quantities V, S_0 and t in the above equations.

The implicit assumption which runs through this type of analysis is that one primary ionization inactivates one molecule. This assumption, first formally proposed by Lea,[3] has been verified for a range of molecular weights from 600 for penicillin,[4] to 6,000,000 for the transforming principle of pneumococcus.[5] The verification consists in showing that the molecular weight of the irradiated material, as found from its sensitive volume, agrees with values obtained by older methods of determining molecular weights. Typical inactivation data taken from the work of Smith[6] for the enzyme desoxyribonuclease are shown in FIGURE 1. The relative enzymatic activity decreases according to EQUATION 1. The inactivation cross section increases with the rate of energy loss of the incident particles. An example of the type of analysis which may be used is shown in FIGURE 2, where the inactivation cross section for DNA-ase (accepted molecular weight 63,000*) is plotted as a function of the energy loss of the incident particle.[6] Electron inactivation data indicate a molecular weight of 63,000, and give the slope at the origin by use of EQUATION 3. The solid curve through the experimental points obtained with deuterons of different energy is of the form of EQUATION 2, and has been drawn for a spherical molecule of radius 29 Å. At high rates of energy loss, the inactivation cross sections are somewhat too large because of inactivation by energetic secondary electrons.[2] Thus the data for DNA-ase are consistent with a spherical molecule of molecular weight 63,000. Similar data taken by Pollard, Powell, and Reaume[7] on invertase indicate that the molecule whose accepted and radiation molecular weights are 120,000 is not spherical, but must be regarded as a cylinder of axial ratio roughly 2:1. The limiting cross section for such a molecule, as shown in FIGURE 3, is greater than the volume of an equivalent sphere. Similar reasoning applied to the transforming principle of pneumococcus indicates that it behaves radiationwise as a long thin cylinder, with axial ratio 80:1.[5]

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* Values for accepted molecular weights may be found in an article by J. T. Edsall in *The Proteins*, IB Academic Press, 1953, or in the other references cited in this article.

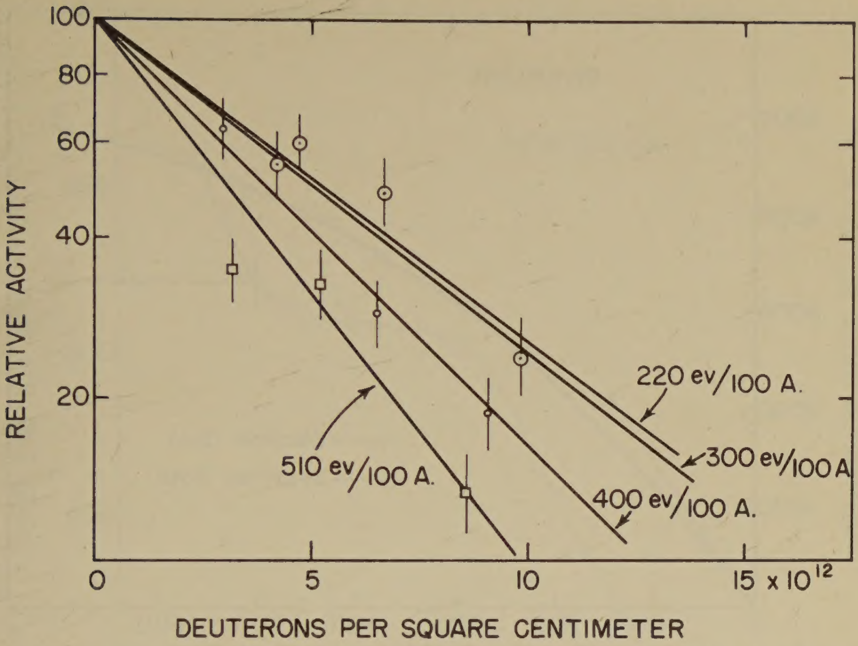

Figure 1. The relative activity of DNA-ase,[6] on a logarithmic scale, versus the number of incident deuterons/cm.,[2] for deuterons of different energies and hence different rates of energy loss.

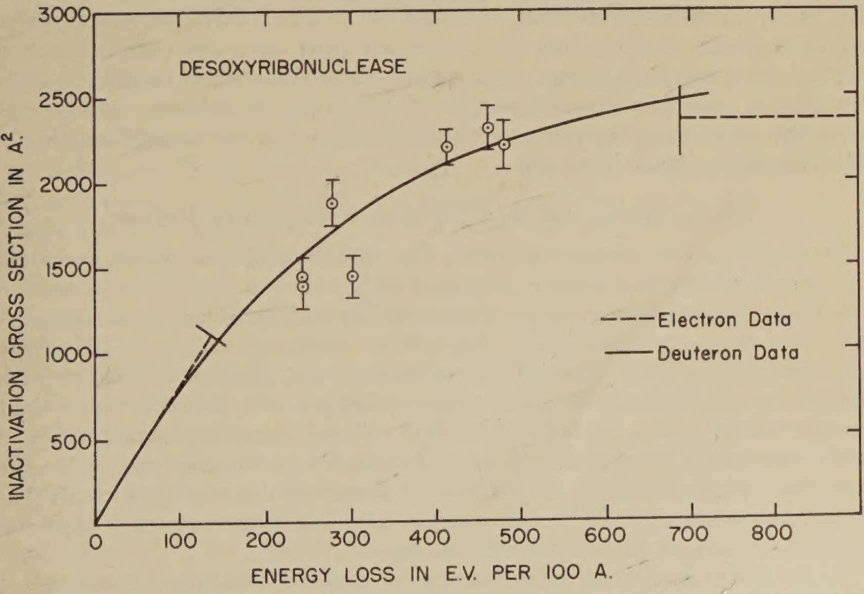

Figure 2. The cross section for DNA-ase inactivation[6] versus the rate of energy loss. The broken horizontal line indicates the cross section expected for a spherical molecule.

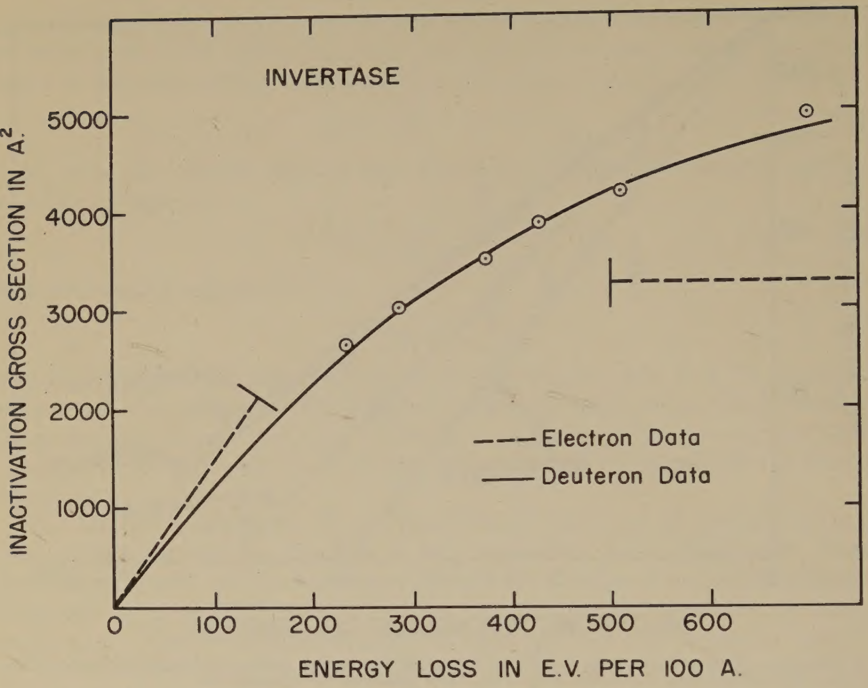

FIGURE 3. Data similar to those of FIGURE 2 for invertase.[7]

The simple generalization—one primary ionization, one molecule—which the above cases illustrate does not hold for others. Some molecules show larger molecular weights than the usually accepted ones, and other molecules act too small from a radiation standpoint. The latter class of deviations from the general case may be explained either by giving the molecule an internal structure, or by assuming that a primary ionization does not damage completely the molecule in which it occurs.

Partial Damage and the Effect of the Resuspending Medium

Hemoglobin is an anomolous case. The solubility of hemoglobin decreases exponentially with irradiation. Appleyard[8] has shown that the cross section for this process depends on the medium in which the hemoglobin is resuspended. FIGURE 4 shows the cross section for 3.7-mev deuterons versus the pH of the resuspending medium. This pH has a large effect. Similar data taken with 2-mev electrons indicate that for a resuspending pH of 5, the molecular weight is about 46,000, while at pH 7 it is 18,000.[9] In both cases, the same number of ionizations have been produced in the molecules in the dry state, and the molecules after irradiation are identical. However, the solubility at pH 7 is much greater than at pH 5, and we conclude that those molecules which remain in solution at pH 7 have been damaged.

A somewhat similar situation exists for catalase. Catalase irradiated at

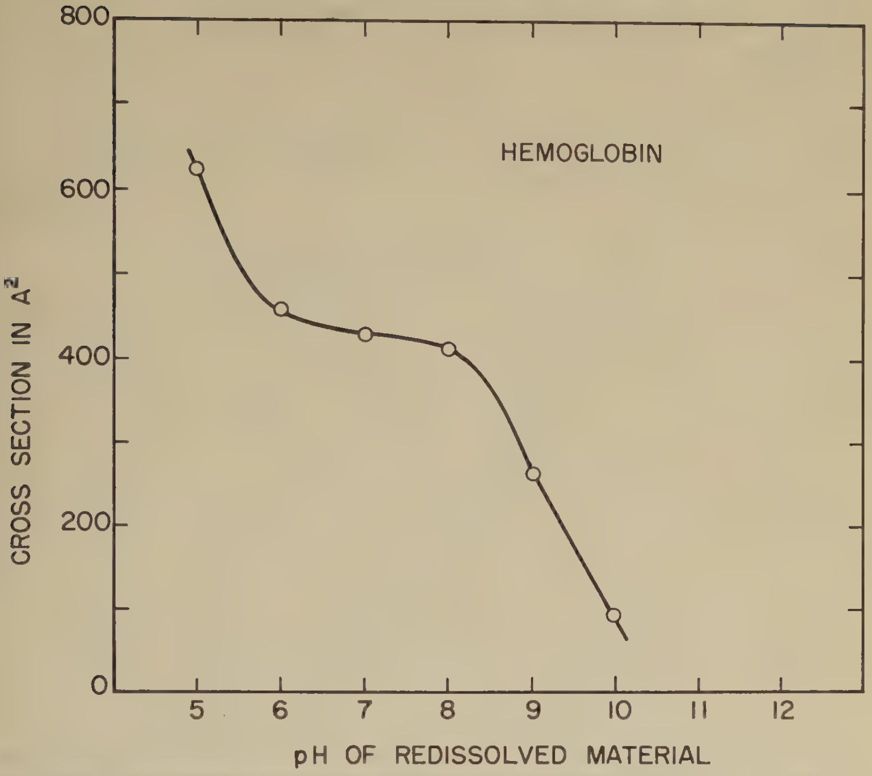

FIGURE 4. The solubility cross section of hemoglobin for 3.7-mev deuterons versus pH of the dissolving medium.[8]

room temperature shows a cross section which corresponds to half its molecular weight. But irradiated samples which still show enzymatic activity are more sensitive to heat inactivation than control samples, indicating that some irradiated molecules had been damaged but were still enzymatically active.[10] A more direct way of showing that some of the enzymatically active catalase molecules have been damaged is to dissolve the samples after irradiation in thiourea instead of water. FIGURE 5 indicates that, for catalase dissolved in thiourea, the cross section is about double that when it is dissolved in water.[11] The factor of two discrepancy between the radiation and accepted molecular weights for catalase disappears upon a proper choice of medium.

By far the best example of partial damage to a molecule by ionizing radiation is the case of bovine serum albumin. The ability of serum albumin to combine with its homologous antibody is destroyed by radiation. If the antigen is irradiated and assayed in the form of a monolayer, it shows a sensitive volume corresponding to a molecular weight of 4,000.[12] If the albumin is irradiated in bulk, it shows a molecular weight of the order of 10 times this figure.[13] The interpretation is that the irradiation of albumin in bulk renders it insoluble. The question of solubility does not arise for monolayers and, in this case, the

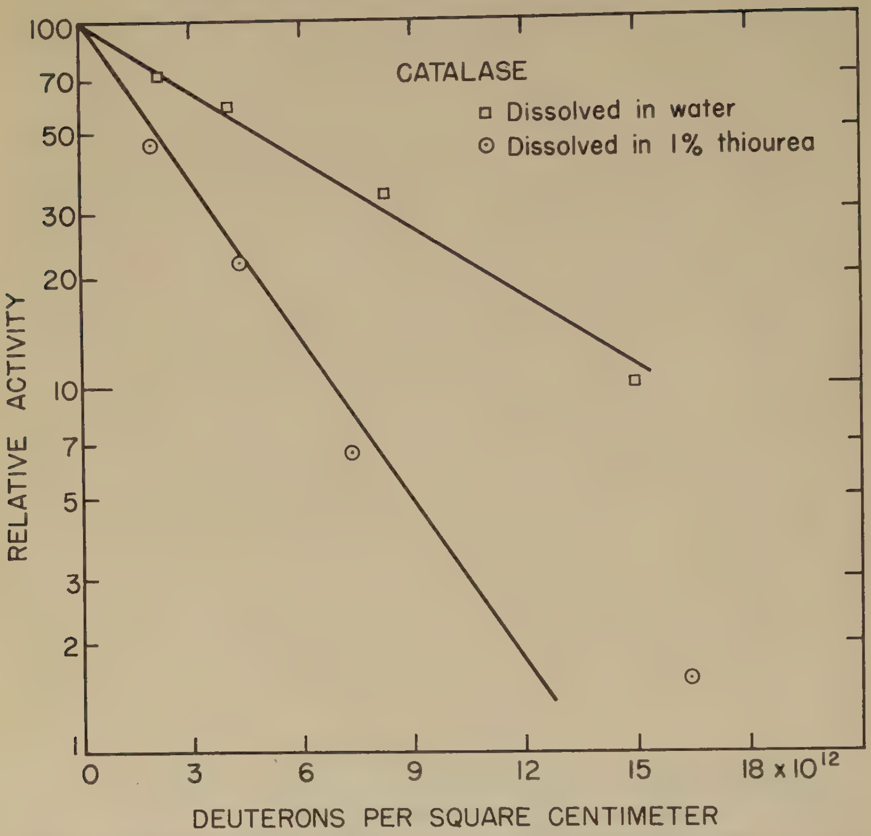

FIGURE 5. The relative activity of catalase as a function of 3.7-mev deuteron dose when dissolved in water and 1 per cent thiourea.[11]

antigenic sites of the molecule itself must be destroyed. The small cross section for the destruction of antibody combining power of the monolayer indicates that the remaining part of the molecule has suffered severe damage without affecting the antigenic site.

Energy Requirements

The fact that not all molecules are necessarily inactivated by primary ionizations leads us to suppose that the observed cross sections may depend upon the energy released within a molecule. We know that this is true in the extreme case of ultraviolet radiation, for here the quantum yields are of the order of 0.01[14] instead of the approximate value of 1 found for charged particle radiation. Molecules such as catalase which show the partial damage phenomenon have still smaller quantum yields. Hutchinson[15] has made a direct determination of the amount of incident energy necessary to inactivate the antibody combining powers of bovine serum albumin monolayers. His results are shown in FIGURE 6, where the cross section is plotted as a function of incident electron

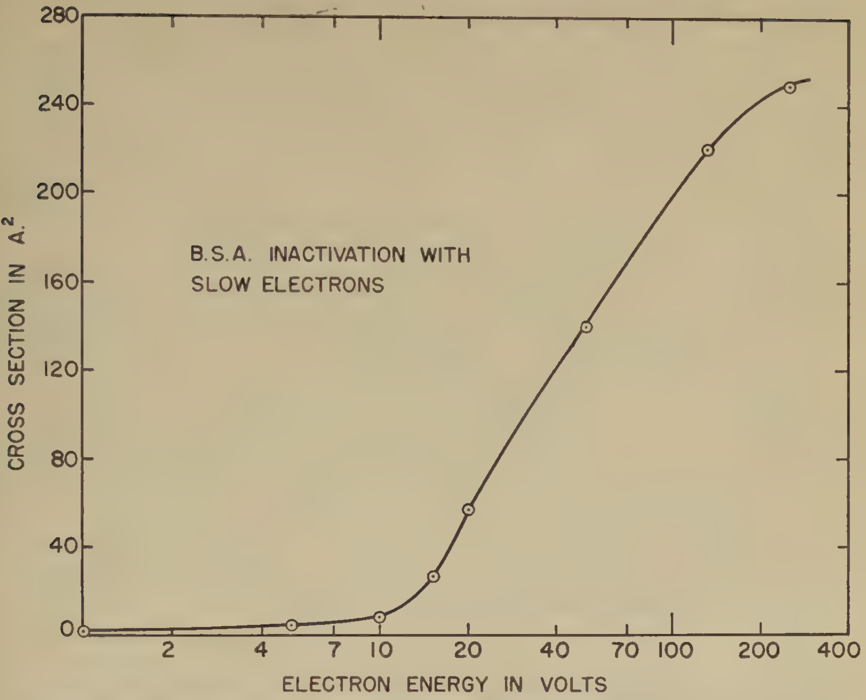

FIGURE 6. The inactivation cross section for bovine serum albumin monolayers as a function of the energy of the incident electrons.[15]

energy. The sharp increase in cross section, in the neighborhood of 10 e.v., corresponds to the point at which ionization rather than excitation takes place.

For molecules such as hemoglobin and bovine serum albumin, which have been assayed for solubility after irradiation, it is found that EQUATION 2 does not describe the variation in cross section with energy loss. Typical results are shown in FIGURE 7 for hemoglobin.[9] The only explanation for a curve of this sort which is concave upwards is that a certain critical energy must be released within the molecule before it is inactivated or, alternatively, that the probability of inactivation rises with energy release (we have assumed up to now that this probability is constant so long as an ionization is produced). At high rates of energy loss, the chance of more than one primary ionization and, therefore, of more energy being liberated in the molecule, is larger. A theory to explain the shape of these ion density curves has been worked out by assuming that a certain critical number of ionizations is necessary to inactivate the molecules.[13] This sort of approach is much easier to carry out mathematically than the more general one which depends on the unknown variation of cross section with energy release. The data for hemoglobin may be fitted by assuming that three simultaneous ionizations, *not* primary ionizations, will cause it to become insoluble. The three ionizations arise from a single primary ionization. If it is required that more than one primary ionization is needed to inactivate a molecule, then the survival curves would not be

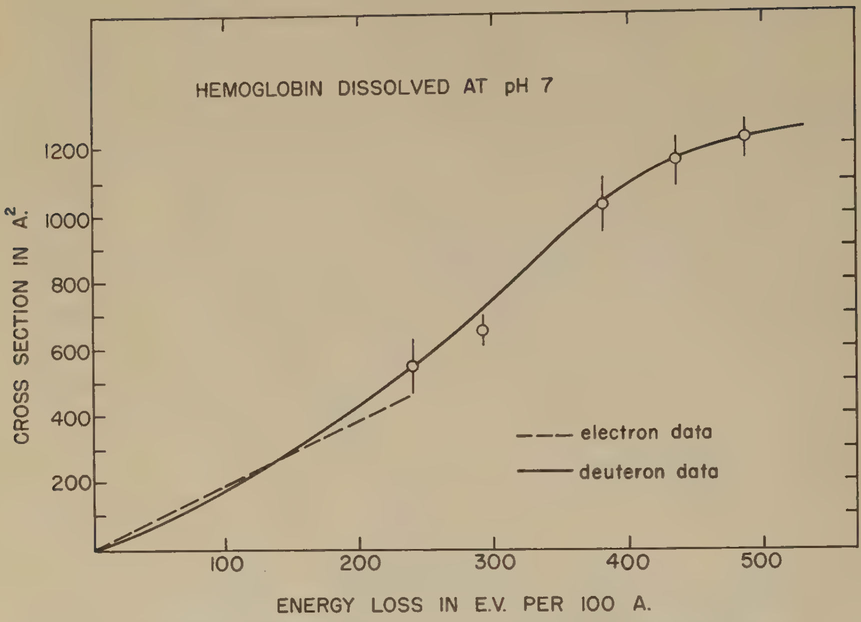

FIGURE 7. Hemoglobin solubility cross section versus rate of energy loss of incident particles.[9]

of the simple exponential form of EQUATION 1, but would show a multiple-hit effect. This effect they do not show. Similar computations for bulk bovine serum albumin[13] indicate that the necessary number of ionizations is 3 and, for alpha amylase, 3 also.[16]

It should not be thought that all cases of enzyme inactivation are just a manifestation of insolubility. For three molecules that have been studied, invertase,[7] catalase,[17] and chymotrypsin,[11] the enzymatic activity has a bigger cross section than solubility. It does appear, however, that the loss of solubility requires a larger energy release than the loss of enzymatic activity. This difference is not surprising, because of the high specificity of enzyme reactions and the low specificity of solubility.

The Effect of Temperature during Irradiation

The fact that, for some molecules, such as catalase and bovine serum albumin, the energy released by an ionization is on the borderline of inactivating them indicates that small changes in thermal energy of the surrounding medium might have a large effect on the radiation cross sections. This large effect is found for all proteins which have been investigated in this way, except hemocyanin.[18] In the latter case, the splitting of the molecule into subunits takes place equally well at room temperature and at 90° K. This splitting, however, is probably a much gentler phenomenon and takes place with much less energy than the inactivation of an enzyme or the fragmentation or loss of solubility of ordinary proteins. In general it is observed that proteins are less sensitive at liquid air temperatures than at room temperature, and, at high temperatures,

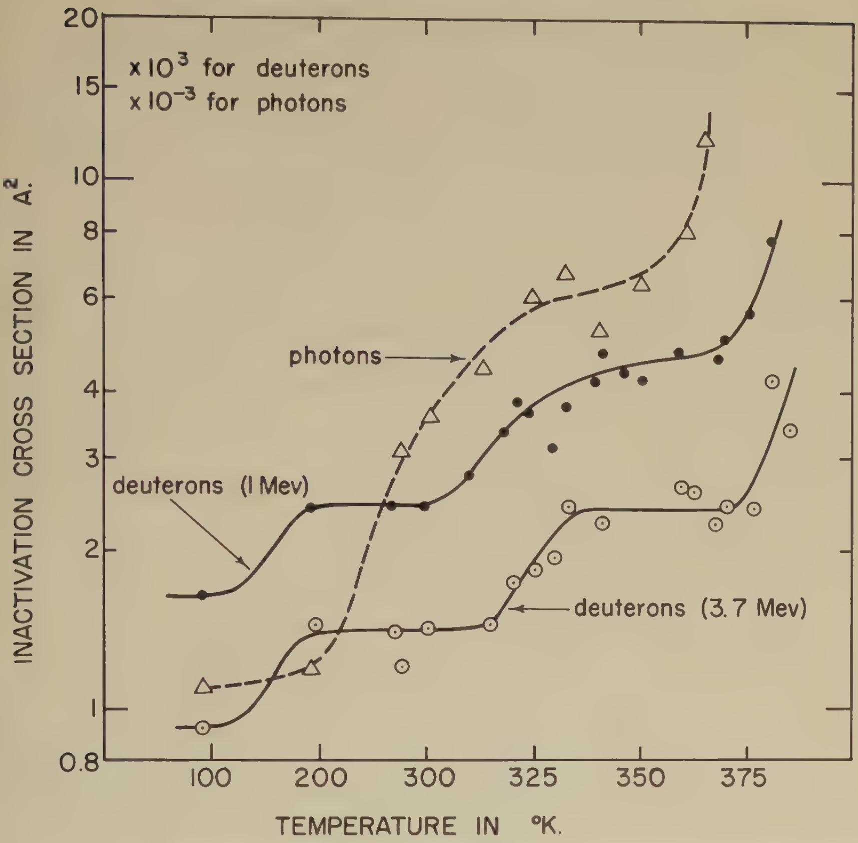

FIGURE 8. The cross sections for inactivation of catalase by deuterons and 2537 Å. photons versus the temperature during irradiation. The temperature scale is not uniform.[19, 20]

the radiation cross section may rise to values greater than those corresponding to the entire molecule. The sort of results which are obtained for catalase[19, 20] are shown in FIGURE 8. The three radiation cross section plateaus indicated in the figure correspond to the inactivation of one fourth, one half, and the whole molecule. The good numerical agreement between these fractions and the fact that catalase has four heme groups, suggests that for catalase the partial damage is total inactivation of parts of the molecule, rather than partial inactivation of the catalytic activity of the molecule as a whole. The too-large value of the charged particle cross section at high temperatures may result from the contribution of excitations to the inactivation process, because, as is indicated in FIGURE 8, the ultraviolet cross section is rising very rapidly in this region. At these temperatures, the rate of heat inactivation is beginning to become important. Any small additional amount of energy transferred to the catalase molecule from neighboring molecules might also inactivate it. At higher rates of energy loss, the cross-section plateaus occur at lower temperatures. Similar results hold for invertase.[7] This result indicates the close connection between the partial damage effect, the temperature effects, and the energy requirements for inactivation. The close connection between these effects is also shown by the sensitivity of the pneumococcus transforming principle at liquid air temperature.[5] The cross section is decreased to about one-third the room temperature value for low rates of energy loss of the incident particles, but is not decreased for a high rate of energy loss.

The Effect of Surrounding Substances

Some molecules show a larger radiation molecular weight than the usually accepted one. Trypsin's 34,000[21] versus the accepted weight, about 20,000, and insulin's 23,000[22] versus the accepted 6,000 or 12,000 are examples. These molecules are known to form dimers and even higher polymers. It is possible that in these cases we are dealing with the transfer of energy from one unit of a polymer to another. An alternative explanation is that an ionization in one member of a polymer causes the entire polymer to become insoluble. Such situations are just the opposite from the partial damage effects discussed above. In the majority of cases, the physical state of the surroundings is unimportant. Crystalline and lyophilized DNA-ase have similar cross sections.[6] When dry invertase was irradiated by low-voltage electrons which could penetrate only one molecular thickness, only one molecular layer was inactivated, even at very high doses; for electrons which could penetrate several layers, only these layers would be inactivated. The results of this type of experiment as performed by Davis[23] are shown in FIGURE 9. They indicate conclusively that, in general, no appreciable amount of energy is transferred from one molecule to another. To explain the trypsin and insulin data, we must take into account the fact that the units of a polymer are bound to one another rather specifically, while the invertase molecules in the experiments cited above are not. To check the hypothesis that a specific binding allows an ionization in one molecule to inactivate another, we cite the following two experiments.

Trypsin and soybean trypsin-inhibitor have been irradiated together.[24] A solution containing the two molecules was dried down at a pH such that they were combined. After irradiation, the samples were dissolved at an acid pH where the inhibitor and the trypsin were uncombined. The amount of trypsin remaining could then be determined. The cross section for the trypsin found in this fashion corresponded to a molecular weight of 30,000. The results of this experiment are not clear-cut because, while the figure 30,000 may correspond to the combination of a monomer of trypsin (molecular weight 17,000) and a monomer of soybean trypsin-inhibitor (molecular weight 12,000), we may regard the figure 30,000 as equal within experimental error to the molecular weight of a dimer of trypsin. The latter explanation is improbable, since it implies that the enzyme and its inhibitor were not combined in the ratio of one trypsin to one soybean trypsin-inhibitor, as is indicated by the chemical evidence.

The second system of molecules in combination which has been investigated is that of hyaluronic acid and its enzyme hyaluronidase.[11] For 3.9-mev deuterons, the cross section for inactivation of the enzyme is 1,650 Å.^{2}, while that of its substrate (measured by its ability to clot an albumin solution) is 3,100

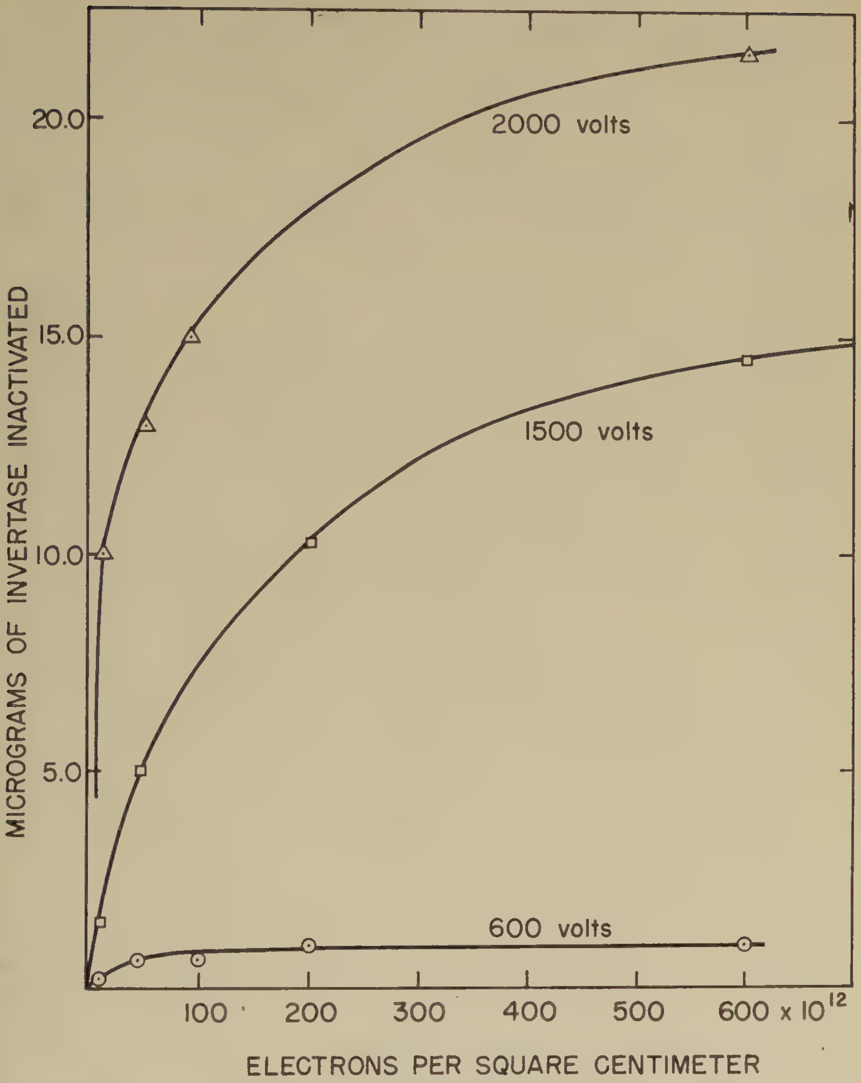

Figure 9. Amount of invertase inactivated versus the dose of incident electrons for different energy electrons.[23]

Å². If enzyme and substrate are allowed to react and dried rapidly, it is found that the cross section for inactivation of the enzyme is larger than that for the enzyme alone, and may indeed approach a value of 4750 Å.², as shown in FIGURE 10. This large cross section is due to the specific combination between enzyme and substrate. Digested substrate or other mucoids do not produce this effect. It is concluded that an ionization in one molecule may very well inactivate another to which it is specifically bound. This conclusion is not surprising in view of the finding by Alexander and Charlesby[25] that energy resulting from ionizations can travel down hydrocarbon chains.

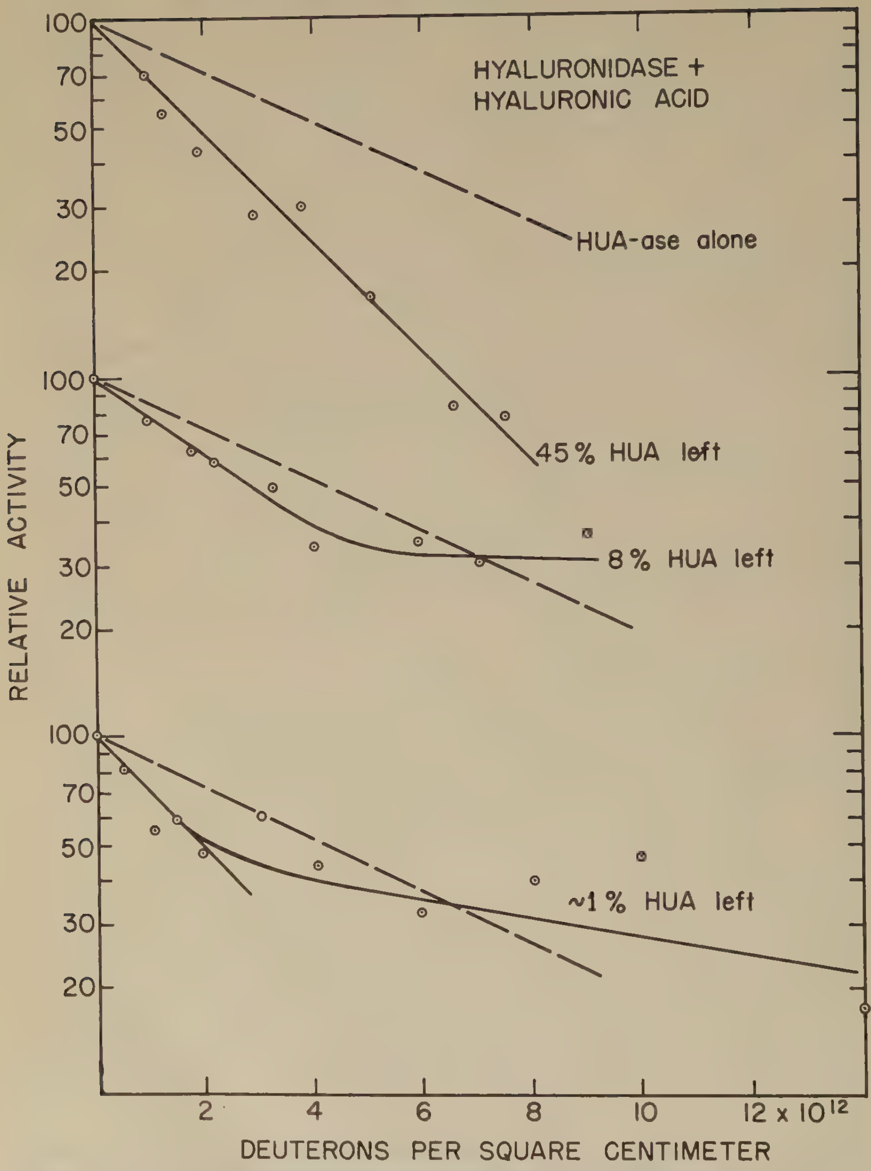

FIGURE 10. The activity of hyaluronidase versus incident dose of 3.9-mev deuterons. The enzyme was irradiated in the presence of various amounts of the undegraded substrate, hyaluronic acid.

Conclusion

The *direct* action of fast charged particles on large molecules is such that, on the average, one primary ionization inactivates the specific function of the molecule. Exceptions to this general rule arise because some molecules may suffer only partial damage as a result of a primary ionization, require more than one simultaneous ionization to be inactivated, or may be inactivated by a primary ionization in a neighboring molecule with which they specifically interact.

Acknowledgments

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Discussion of the Paper

Doctor Kurt Salomon: Did you investigate carbon monoxide hemoglobin?

Doctor Setlow: The work of Appleyard[8, 9] was on carbon monoxide hemoglobin.

Doctor Salomon: Did you investigate hemoglobins of larger molecular weight than mammalian hemoglobin; for instance, the erythrocuorin of the earth worm or other invertebrate hemoglobins?

Doctor Setlow: Hemocyanin (molecular weight 7,000,000) was irradiated by alpha particles by Svedberg and Brohult.[18] They found that one alpha particle passing through a molecule split it into subunits.

BACTERIAL VIRUS: RADIATION AND FUNCTION*

By D. J. Fluke and E. C. Pollard
Brookhaven National Laboratory, Upton, Long Island, N. Y.; and Yale University, New Haven, Conn.

One of the materials most extensively studied with the dry state ionizing particle approach described in the preceding papers has been T-1 bacteriophage. It was one of the earliest materials subjected to cyclotron bombardment in the Yale biophysics group (Pollard and Forro, 1951) and a number of workers associated with the group have returned to it from time to time (Pollard and Jane Setlow, 1954). One reason is that investigation of various functional properties of a single material is relatively easy for bacteriophage and, among the T-series coliphages, T-1 is the most easily handled dry. Another reason is that the bacteriophage work, as represented to a large extent by T-1, does not fit the one primary ionization-one inactivation conclusion stated in the preceding paper by Doctor R. B. Setlow for many other materials. T-1 is one of the exceptions, and we have kept coming back to it to see more clearly why it is exceptional.

The data presented in this paper are results obtained by the first author in a series of bombardments of T-1 at five values of linear energy loss (effectively LET, or linear energy transfer), tested with respect to three virus functions. In part, these results repeat those of the second author.

T-1 bacteriophage is a virus of the host bacterial strain *Escherichia coli B.* The virus was prepared as a filtered broth lysate titering about 10^{11} per ml. and was irradiated in .05 ml. amounts dried on ½″ diameter glass cover slips. The cover slips were independently exposed in an evacuated bombardment chamber to measured fluxes of the various particle radiations, after which they were dropped into broth for resuspension of the virus material. Assays of various functional properties were performed.

The radiations used were 2-mev electrons from a Van de Graaff generator, made available to us in the Brookhaven Chemistry Department by Doctor A. O. Allen, 3.5 and 1.1-mev deuterons from the Yale cyclotron, and 6.6 and 3.5-mev alpha particles from the same machine. The lower energy for each particle was obtained by placing aluminum foils of measured mass per unit area over the coverslips during bombardment. The bombardment chambers and experimental details have been described (Pollard and Forro, 1951; Pollard, Buzzell, Jeffreys, and Forro, 1951). Briefly, the technique involves spreading the beam out uniformly over a known area by a suitable arrangement of upstream apertures and scattering foils. A shutter starts and stops the irradiations and the incident beam is collected and measured as a current.

The linear energy loss for each of the radiations is calculated from the appropriate Bethe-Bloch formula (Siri, 1949), assuming a representative protein composition $C_4H_8NO_2$ and density 1.35 gm./cm.^{3} Similar calculations for a DNA composition give about 15 per cent higher figures for linear energy loss.

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* Research carried out at Brookhaven National Laboratory under the auspices of the United States Atomic Energy Commission.

The density used for DNA was 1.65 gm./cm.[3]. At the same density as for protein, the linear energy loss in DNA is about 5 per cent lower than for protein. The linear energy losses applied range from 2.9 e.v./100 Å. for the electrons to 1600 e.v./100 Å. for the slower alpha particles.

The three virus properties tested were: (1) ability to multiply in host cells; (2) ability to kill hosts cells; and (3) one-step growth behavior of surviving virus. The first property is shown by formation of plaques on agar plating with the host bacteria. The various bombarded samples are appropriately diluted in broth, and aliquots are plated for plaque counting. Results for the 2-mev electrons are shown in FIGURE 1 and, for the other particles, in FIGURE 2. Survivals are plotted on a logarithmic scale against dose in particles per unit area. The dose-effect curves are straight lines. The slope increases with higher linear energy loss.

The bacterial killing ability (BK) of the virus was measured by exposing *E. coli* to the irradiated virus and measuring the rate of decrease of bacterial

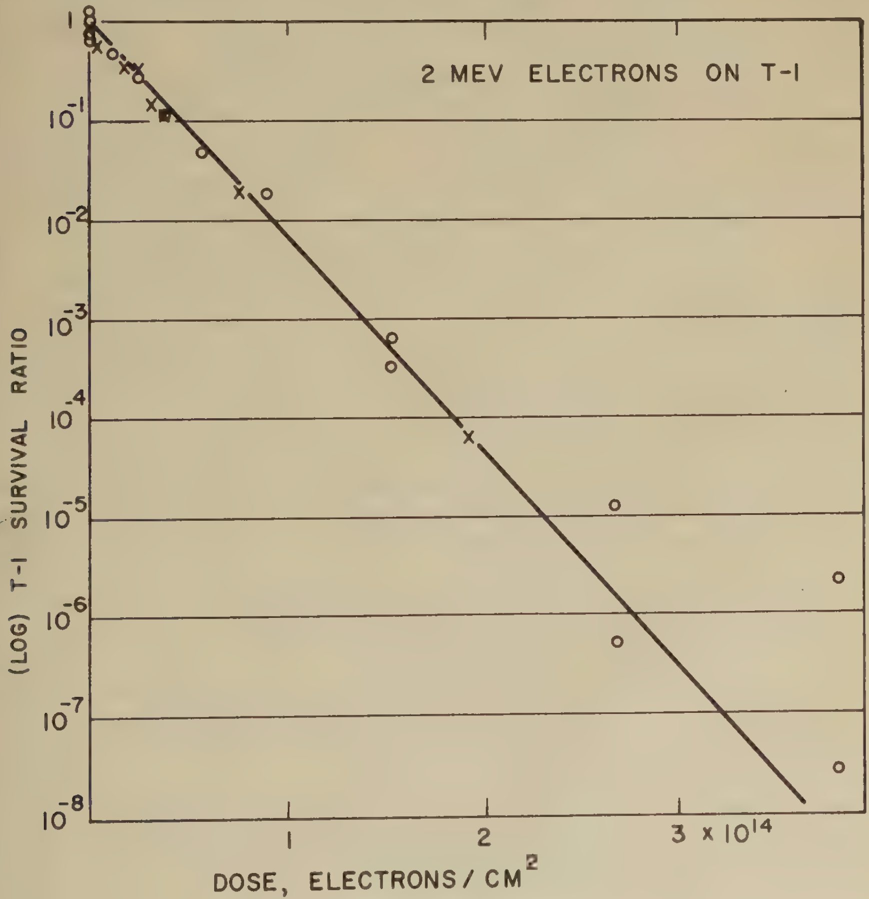

FIGURE 1. Survival of T-1 multiplying ability for various doses of 2-mev electrons.

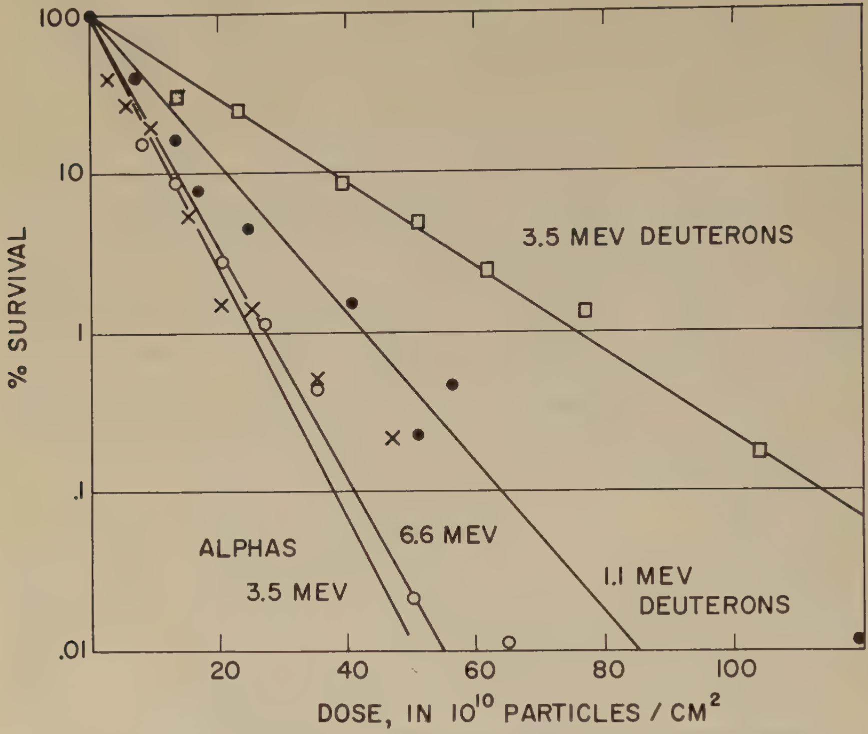

FIGURE 2. Survivals of T-1 multiplying ability for bombardment by various ionizing particles.

survival. Because of the short latent period of T-1 (13 minutes at 37° C.), the more usual method of limiting bacterial survival could not be conveniently used with log phase bacteria.

The dose-effect curves for BK are also logarithmic, as may be seen in FIGURE 3 for 2-mev electrons and, in FIGURE 4, for 1.1-mev deuterons.

The cross section for BK is one third that of multiplying ability at lower linear energy loss. Watson (1950) observed a 1:2.5 ratio for BK to survival for T-1, with 1:3 for T-even phages. At higher linear energy loss, the BK cross section increases faster than the cross section for multiplying ability. By cross section is meant the probability of inactivation by the ionizing particles, measured as the reciprocal of the dose corresponding to 37 per cent survival of activity, as discussed in the preceding papers.

The cross sections for multiplying ability and BK are plotted against linear energy loss in FIGURE 5. The electron data are represented as slopes at the origin (dashed lines). A number of features may be seen. The cross section for plaque survival (multiplying ability) does not saturate until quite high linear energy loss. The saturation value corresponds to an area somewhat less than the silhouette of the virus head, 50 mμ in diameter (Williams, 1953). Davis (1953) has shown by low energy electron bombardment that the virus tails are insensitive to inactivation.

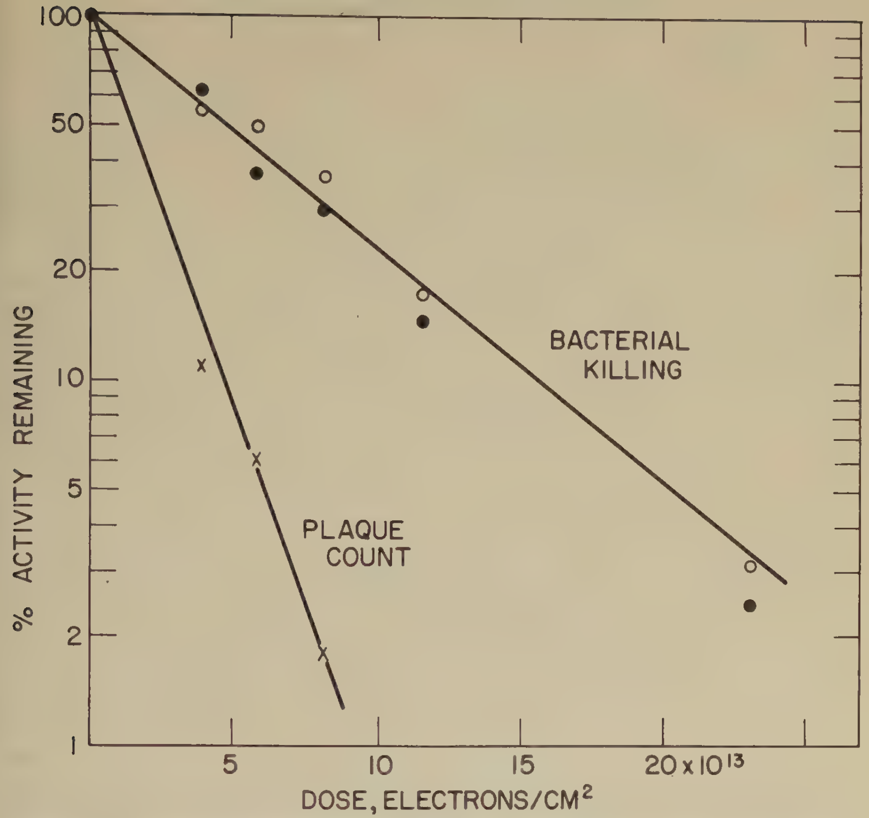

Figure 3. T-1 multiplying ability and bacterial killing ability compared for 2-mev electron bombardment.

The curves show upward concavity at the origin. For both plaque survival and BK, the deutron cross sections lie well above the extrapolations from the electron cross sections. The curves cannot be fitted to the $1 - e^{-x}$ function representing saturation of cross section on a one primary ionization-one inactivation hypothesis. The behavior, at the origin, is taken to mean that some events along the particle tracks cooperate to produce inactivation and hence are insufficient for inactivation on isolated occurrence.

Data on linear-energy-loss dependence of plaque survival cross section for T-1 obtained by the second author have been shown in the first paper of this conference. Figure 5 is in general agreement with this result. The second author has analyzed his result with respect to a target theory allowing for a range of multiplicities for cooperation between events along the track, with indication that, on the average, three or four ion pairs are required within a sensitive locale for inactivation to occur.

The electron survival data may also be analyzed on a volume basis because the particle tracks are much closer together than events along the individual tracks. The survival curve is single-hit, but about 40 primary ionization events

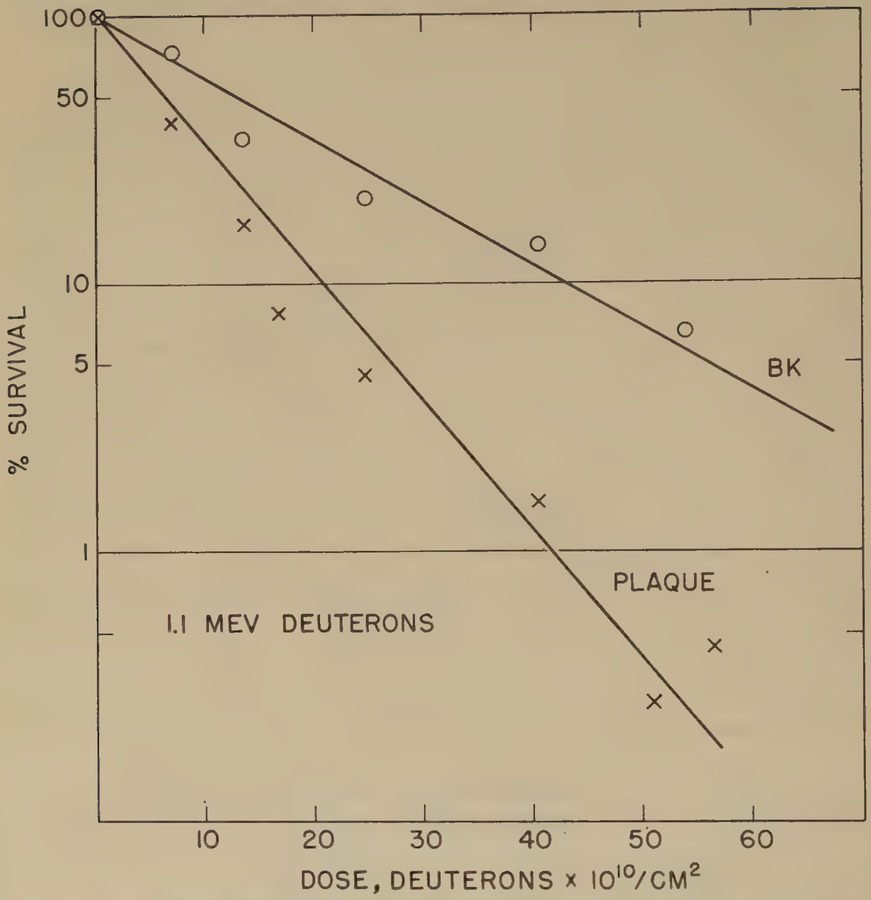

FIGURE 4. T-1 multiplying ability and bacterial killing ability compared for 1.1-mev deuteron bombardment.

occur in the bacteriophage particle, on the average, before the one inactivating event occurs. Even excluding the behavior at the origin in FIGURE 5, on the assumption that one primary ionization occurring within a sensitive volume can inactivate, the sensitive volume would have to be $1/40$ the whole particle size, have a thickness of only about 10 Å., and yet be distributed through nearly the whole silhouette of the virus head.

It seems more reasonable to conclude that more than one average primary ionization is required for inactivation. The inactivating occurrence in the electron bombardment waits for simultaneous events close together in the over-all random distribution. The cooperating events have to result from the same incident electron, or else the dose-survival curve could not be single-hit. The gain in efficiency of inactivation for the same energy per bacteriophage particle for deuterons over electrons confirms this view.

The relation of X-ray inactivation and electron inactivation of T-1 appears

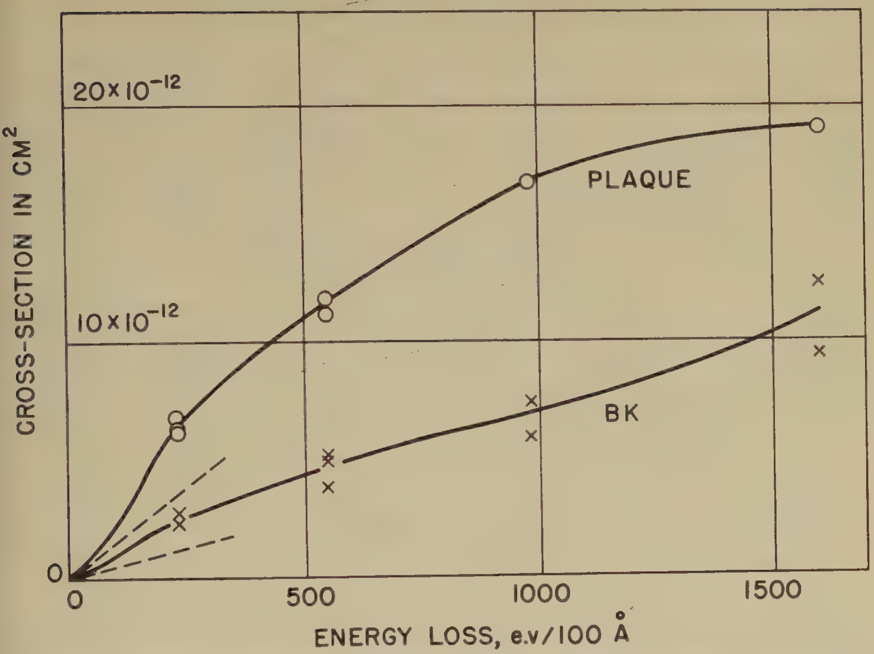

FIGURE 5. Multiplying ability and bacterial killing ability cross sections as functions of linear energy loss of the bombarding particles.

to point up further the enhancement of the inactivation with moderate increase in linear energy loss. The 37 per cent survival electron dose represents about 750,000 rep., while typical X-ray doses are much lower. Pollard and Forro (1951) show a 37 per cent survival dose of about 220,000 r. for X rays on dry T-1.

The BK cross section has not saturated at the highest linear energy loss applied. The linear energy loss dependence of BK cross sections through the lower part of the curve appears to be the same as for plaque cross section but, at higher linear energy loss, something different is happening.

The bombarded virus samples have also been examined with respect to one-step growth behavior. The survivors of various radiation doses have been diluted to the same surviving plaque titer in broth and further diluted, all by the same factor, in phosphate buffer. Log phase broth cultures of *E. coli* were washed by twice centrifuging and resuspending in phosphate buffer. The buffer was 1/150 M in order to provide a suitable monovalent cation concentration for T-1 adsorption (Puck, Garen, and Cline, 1951). The cells and diluted virus were mixed at 37° C. in concentrations appropriate for rapid adsorption with single infection and, after three minutes, were diluted into nutrient broth at 37° C. Timed aliquots were plated for plaque counts. Results for survivors of 2-mev electron bombardment are shown in FIGURE 6, for 3.5-mev deuteron bombardment in FIGURE 7, and for 3.5-mev alpha bombardment in FIGURE 8. Each plot is a representative simultaneous run of several virus samples on por-

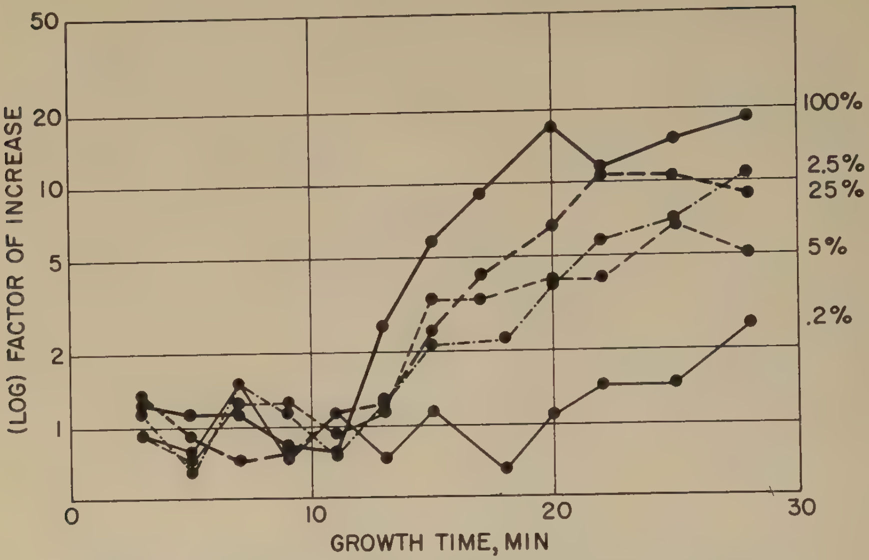

FIGURE 6. Gr wth curves of T-1 inactivated to various survival by 2-mev electrons.

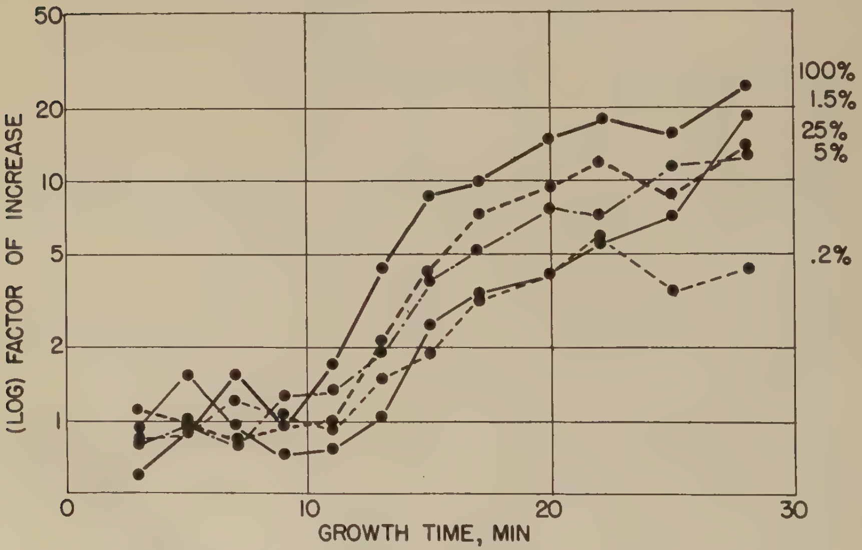

FIGURE 7. Growth curves of T-1 inactivated to various survival by 3.5-mev deuterons.

tions of the same *coli* preparation. The starting level of plaque counts in each series is from about 20 to 40 plaques per plate, and all counts have been plotted on a logarithmic scale as ratios to the average plaque count during latent period. The fluctuations in each curve are of statistical origin. Near the right margin is shown the plaque survival level of each bombarded sample and control before dilution.

The survivors of 2-mev electron bombardment show increasing growth

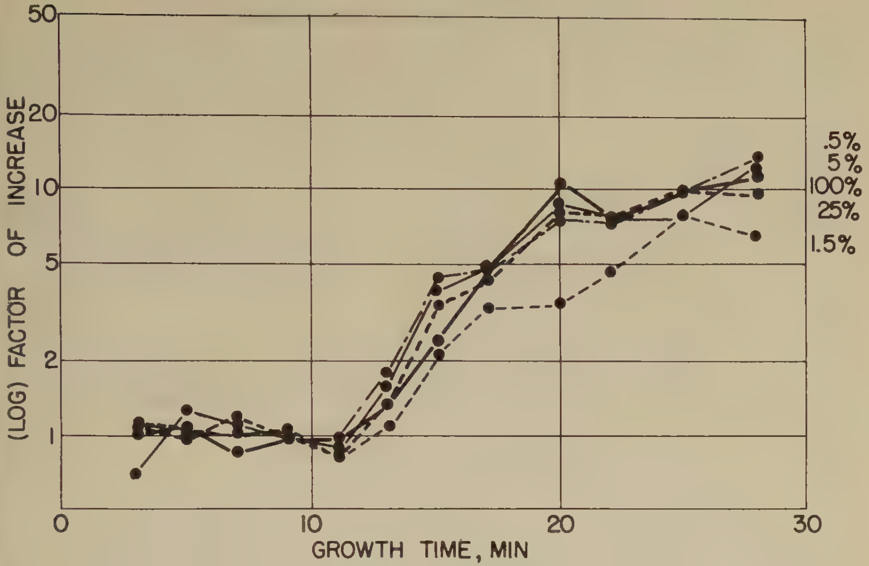

Figure 8. Growth curves of T-1 inactivated to various survival by 3.5-mev alpha particles.

cycle deterioration with increasing dose. Long before visible plaques are seen, the progeny of such damaged survivors must return to normal growth cycle behavior. But the first cycle shows a lengthening of minimum latent period and some combination of either an increased dispersion in latent period

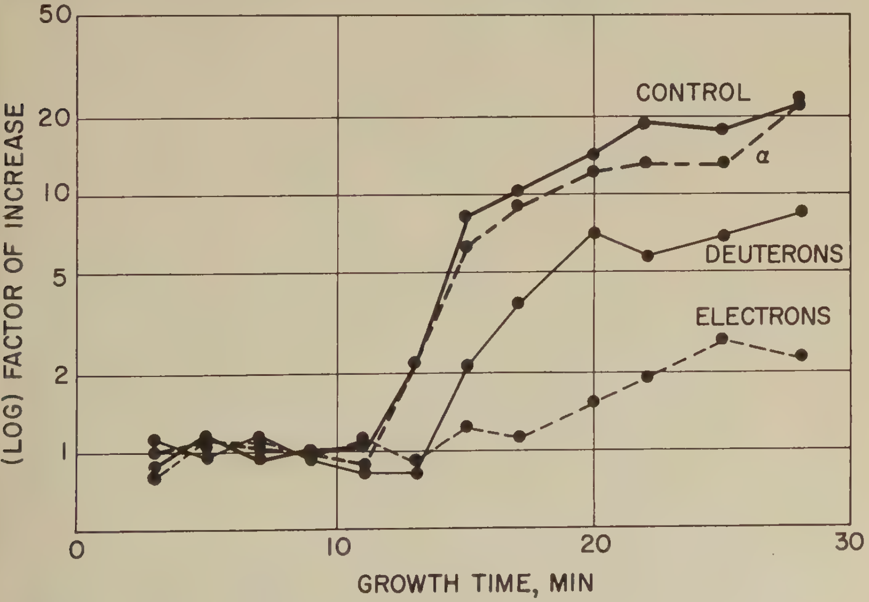

Figure 9. Comparative growth curves for T-1 inactivated to about 0.2 per cent survival by various particle bombardment.

or a decreased burst size. The deuteron survivors show the same effect in decreased amount, and the survivors of 3.5-mev alpha particle bombardment show no significant damage to the growth cycle behavior. The 1.5 per cent survival material is somewhat low in FIGURE 8, but the 0.5 per cent survivors are indistinguishable from controls. The various curves shifted their relations within about the same spread on repetition.

One repetition assay is shown in FIGURE 9, where material of about 0.2 per cent survival from each of the same three bombardment series was run simultaneously with included control material. Clearly, there is extensive partial damage to survivors of bombardment at low linear energy loss, and not for survivors at high linear energy loss.

The growth cycle result confirms observations by the second author on T-1 latent period (Pollard, Adams, and Robbins, 1954), which is increased by bombardment with 3.8-mev deuterons and less so at higher linear energy loss. The observations involve quite different conditions and methods, and sample the bombarded viruses in a different manner.

Discussion

A dry T-1 bacteriophage particle is not ordinarily inactivated by a single ion pair, or even by an average single primary ionization occurring anywhere within it. The idea of a more limited sensitive volume inactivable on the same basis is unsatisfactory because the low linear energy loss result requires so small a volume (1⁄40 the phage volume), and the high linear energy loss result requires so large an area (most of the virus head). Inactivation is progressive. Active phage can show partial damage and inactive phage can show partial function. These facts have been at hand for some time.

A more recent finding (Davis, 1953), with slow electrons of various range, shows that the physical events associated with virus inactivation must occur fairly deep inside the virus, so deep that none can have effect in the tail. The target area at high linear energy loss is not completely consistent with slow electron evidence for an insensitive outer layer, but at least we can conclude that the inactivable part of the virus is spread across all the area in the virus head that Davis' result will allow.

The inactivating events for T-1 can be divided into two classes on the basis of an energy threshold. The first class of events, those below threshold, can cripple the next virus growth cycle without causing failure to produce some active progeny. Such events are additive with respect to crippling, but cannot add up to complete inactivation. The conclusion is based on the rigorous single-hit curve for inactivation even at lowest linear energy loss, where subthreshold events are most predominant. If subthreshold events occur close enough together and simultaneously (same particle track), they become indistinguishable from events of the second or above-threshold class. Apparently subthreshold events are vulnerable on occurrence, but settle into a relatively stable long-term condition.

The lack of effect on growth curves after high linear energy loss bombardment leads to two conclusions. No substantial part of the virus particle can be accessible to crippling action without also being potentially inactivable; the same material is probably involved. Second, not much of the damage produced at high linear energy loss would appear to be due to delta rays, or effects on growth curves should be seen.

The similarity in energy loss dependence at low values for bacterial killing and virus-multiplying ability suggests that the same material and the same general mechanism of action are involved. The factor of three difference in cross section might at first indicate that an average of three hits are required to reduce the pertinent material to fragments small enough to be ineffective in stalling the bacterial mechanism. The observed single-hit dependence on dose makes it more likely that only one third of the potentially inactivable material is directly involved in bacterial killing. The factor of one third increases at high linear energy loss. It may be that very large energy losses in the section not involved in bacterial killing can result in migration of energy into the section more directly involved in the killing effect.

The above facts and conclusions ought to make sense in relation to the considerable array of other knowledge about bacteriophage composition and function. A reasonable picture of the bacteriophage particle appears to be that it is a complement of functional DNA (desoxyribose nucleic acid) packaged in a protein coat which serves as the means of reintroducing the DNA into the bacterial cell and probably gives the particle its remarkable stability. The packaging of the DNA may be tight enough to account for the rather high localized energy required for disruption of DNA function.

The simple target theory, which has had relative success with the rest of the work with ionizing particles on dry biologically active materials, has had to be considerably modified to account for T-1. Additional experimental facts have often required changes in the picture instead of confirming what went before; but the deliberately-introduced physical conditions of the experiment favor interpretation by a target theory, and the picture on this basis is a reasonable and informative one.

Acknowledgment

The authors acknowledge helpful discussion with Doctor Frederick Forro and with members of the Yale biophysics group.

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USE OF CHARGED PARTICLES TO MEASURE SKIN THICKNESS AND OTHER SURFACE PROPERTIES

By Franklin Hutchinson
Biophysics Department, Sloane Physics Laboratory, Yale University, New Haven, Conn.

Charged particles, such as electrons or protons, and electromagnetic radiation interact with matter in significantly different ways. The interaction of a photon with an atom characteristically results in a large effect on the photon, either in its energy, in its direction of travel, or both. X-ray photons usually pass through many atoms without any effect. The absorption is purely statistical, and there is always a finite chance that a photon may penetrate any given depth before absorption. Charged particles, on the other hand, typically interact with a much greater fraction of the atoms through which they pass, usually giving up only a small amount of energy at each collision. A particle with more than one or two hundred volts' initial energy will make a number of collisions before stopping. The averaging process, introduced by the relatively numerous collisions needed to stop such a particle, insures that the average distance along the particle track will not vary enormously from a mean value. In particular, most particles will stop within a distance, called the range, which will depend on the type of particle and the energy, and only a minute number will travel even slightly farther than this range.

By using a charged particle beam of controlled energy, it is possible to irradiate the outer surface of a biological organism without producing any direct radiation effects at a depth greater than the particle range. It is the purpose of this paper to discuss some features of this method, and to summarize some recent applications to specific biological systems.

The Method

FIGURE 1 shows schematically the apparatus used to irradiate biological material with a slow electron beam. Ten samples can be placed around the perimeter of the disk and rotated, one at a time, into the electron beam. The dose is measured by the time of exposure and the current arriving at the disk, as measured by the galvanometer. No account is ordinarily taken of secondary electrons, but checks with Faraday cups show that the correction is usually not more than 30 per cent. With various modifications of the electron gun to suit the energy of the electron beam, the apparatus has been used from about two volts to 3500 volts. The currents which have been used lie between .01 and 100 microamperes/sq. cm.

Electrons are not the most suitable charged particles for use as radiation probes, because their small mass lets them be strongly scattered in their interactions with atoms. The total range measured along the actual track is about constant for all electrons of a given energy, but the many sudden changes in direction mean that electrons diffuse different distances into matter before being stopped. There will be a few electrons which, by chance, will be very little deviated from the forward direction at any time, and will consequently penetrate, at most, to a distance substantially the range of the electrons. In short, radiation effects will be a maximum at the surface, and will drop off rapidly with depth. Heavy charged particles such as protons, which are far less likely to be deflected in collisions, would make a better probe, but an electron beam is much easier to obtain.

When the concept of a radiation probe arose, the organisms under consideration were viruses and bacterial spores. The dimensions of interest were thus from tens up to thousands of Ångströms. From existing data, it was possible to estimate that the electron beam energies needed were from hundreds to thousands of volts. Before quantitative work could be done, it was first necessary to measure the actual ranges in matter of electrons of these energies.

Measurement of Electron Ranges

Davis[1] measured the electron ranges by laying down thin layers of an enzyme, invertase, and bombarding these layers with electron beams of various energies. Suppose that an invertase layer 600 Å. thick is irradiated with a beam of 1200 volt electrons intense enough to inactivate all the invertase within the electron range. If an assay of the invertase shows that 50 per cent has lost its biological function, the range of 1200 volt electrons would be deduced as

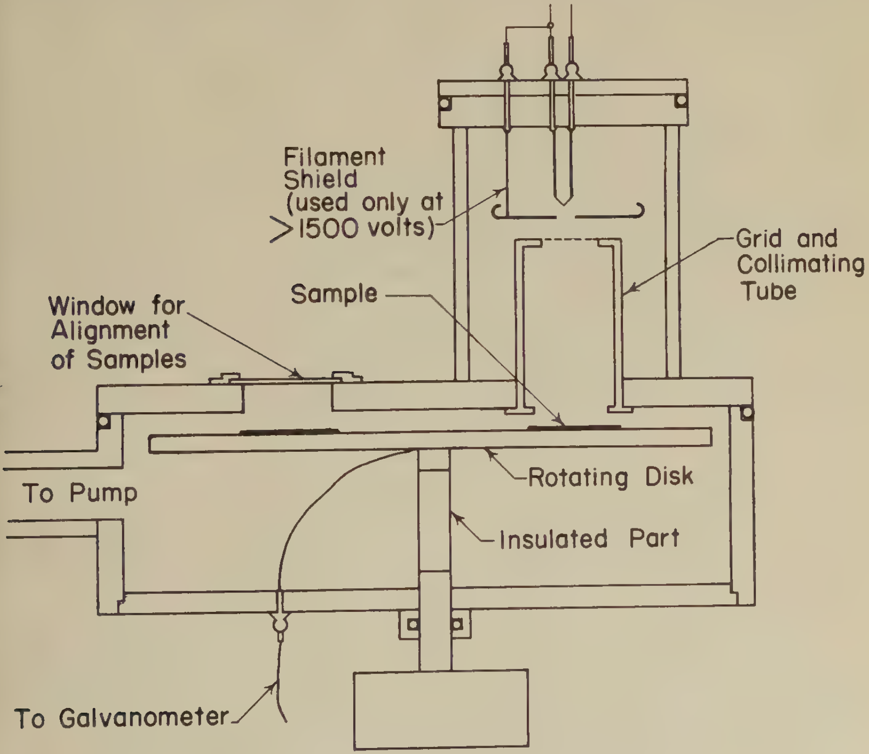
Filament Shield (used only at >1500 volts)
Grid and Collimating Tube
Sample
Window for Alignment of Samples
To Pump
Rotating Disk
Insulated Part
To Galvanometer

Figure 1. Apparatus for irradiation with slow electrons.

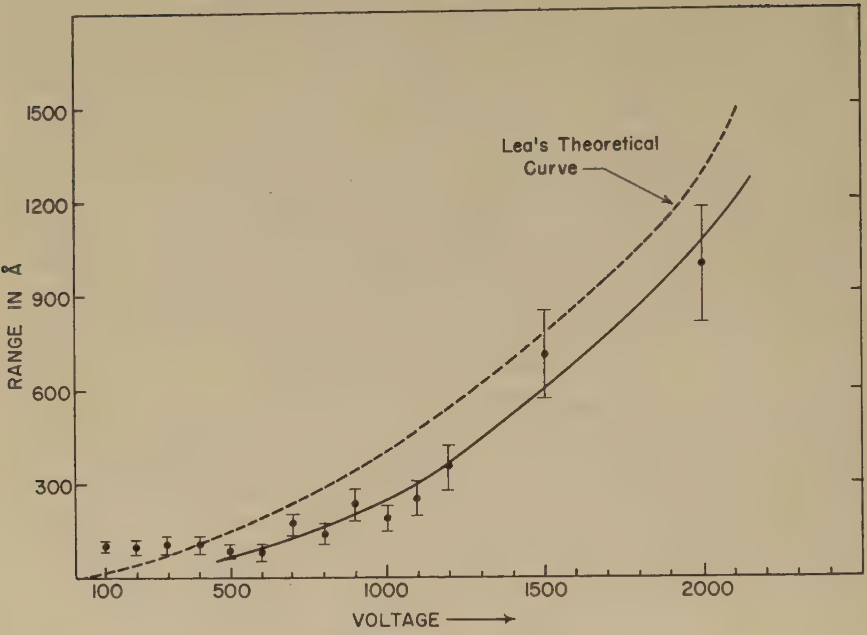

Figure 2. Apparent range of electrons in protein, from Davis.[1]

one half of 600 Å., or 300Å. Figure 2 summarizes the data obtained in this way from 100 to 2000 volts.

Above 600 volts, the observed range follows a curve similar to that obtained from theoretical calculations,[2] but lying about 150 Å. below. The curve below 600 volts is interesting, showing a constant amount of inactivation corresponding to about 90 Å. range for all energies. Ninety Å. is in reasonable accord with the maximum dimensions of the invertase molecule. The implication is that, for all energies below 600 volts, the electron beam will burn off the top layer of molecules, but the effects will not penetrate down into the second layer. This is a most important confirmation of the validity of the concepts underlying the use of a radiation probe of restricted range.

Applications to Viruses

T-1 bacteriophage attacks the bacterium *Escherichia coli* and has a head 500 Å. in dimensions, and a tail 1500 Å. long. From other studies,[3] it is believed to consist of a core containing essentially all the virus nucleic acid surrounded by a protein coat.

If dried preparations containing less than a microgram/sq. cm. of solids of these viruses are irradiated with electrons of various energies, logarithmic inactivation curves are obtained.[4] If the slopes of these straight lines, as measured[5] by the equivalent "cross section," are plotted as a function of electron energy, the curve shown in figure 3 is obtained. A large cross section indicates high radiation sensitivity, and vice versa.

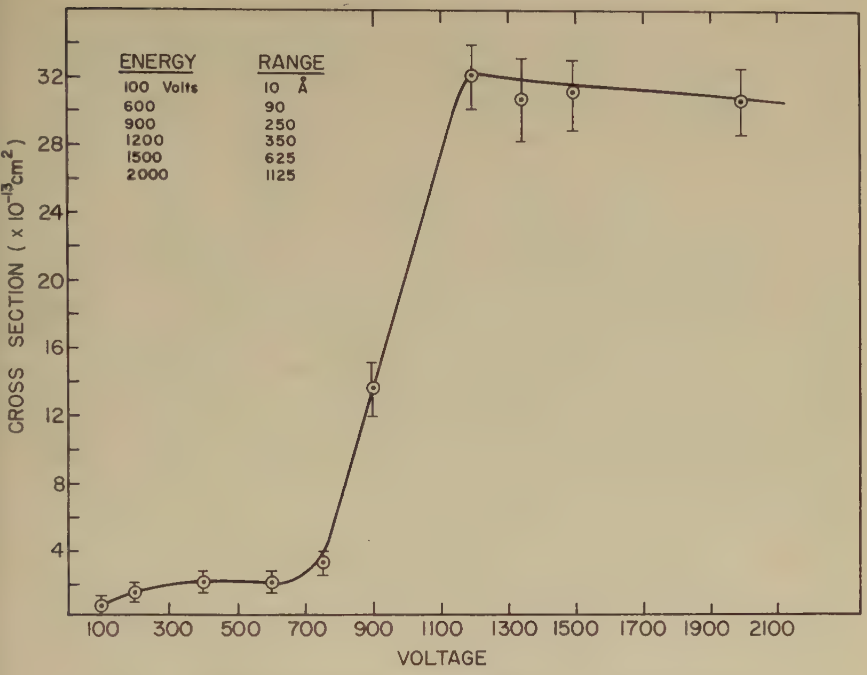

FIGURE 3. The inactivation of T-1 bacteriophage with slow electrons, from Davis.[4]

The most conspicuous feature of the curve is the abrupt rise in cross section for 700-volt electrons. It is quite certain that this rise is associated with the penetration of the protein coat by electrons of this energy. It may be concluded that the protein coat is relatively unimportant to the process of reproduction of the virus, and that this protective coat is about 125 Å. thick. This thickness agrees well with the thickness for protein coats from similar phage found from electron micrographs.[6] The explanation of the plateau running from 100 to 700 volts is more obscure. Probably, at least part is the effect of radiation on the attachment site of the virus, which is the region which fastens to the surface of the bacterium under attack.

It has been shown that viruses normally have a fixed time, or "latent period," between the moment of attachment and the time the burst of new virus particles escape from the bacterial cell. This latent period, however, can be increased by the action of ionizing radiation.[7] It does not appear to be a genetic effect, but occurs in only the first generation. The question is whether the effect is associated with the nucleic acid core or with the protein complement of the virus particle.

FIGURE 4 shows the result on the latent period of irradiating T-1 with 400 volt electrons, which are stopped completely in the protein coat, and with 1200 volt electrons, which penetrate well in to the nucleic acid core.[8] It is seen that the more energetic electrons are much more effective (note that the scale of the abcissa is different in the two graphs), indicating that the increased latent period is probably associated with effects on the nucleic acid. The cause of the small effect of 400-volt electrons is not clear, but it is possible that it is those few electrons which leak through the protein barrier because of the statistical chance of making an unusually small number of inelastic collisions in traveling a distance equal to the range.

Limitations on the Targets

With these examples of the method in mind, it might be well to consider the limitations on the targets which can be irradiated. In the first place, it is necessary that the target material be spread out in a layer sufficiently thin so that no target unit is hidden behind another. If the problem is one of irradiating a number of viruses, say two or three hundred, as above, the restriction is not at all serious. However, even in irradiating plant viruses, where some conventional assays require the order of a microgram of virus, the limitation is serious and, if it would be desired to have enough to do some chemical analysis on the irradiated material, for example, the requirement would be essentially prohibitive.

The effect of debris and of other unwanted material often found in preparations can be minimized by using a sufficiently small sample to give an average thickness of solid material many times thinner than the least dimension of the organism under study. Even here, the debris may be drawn up around the organisms by the action of surface tension during the evaporation of the water.

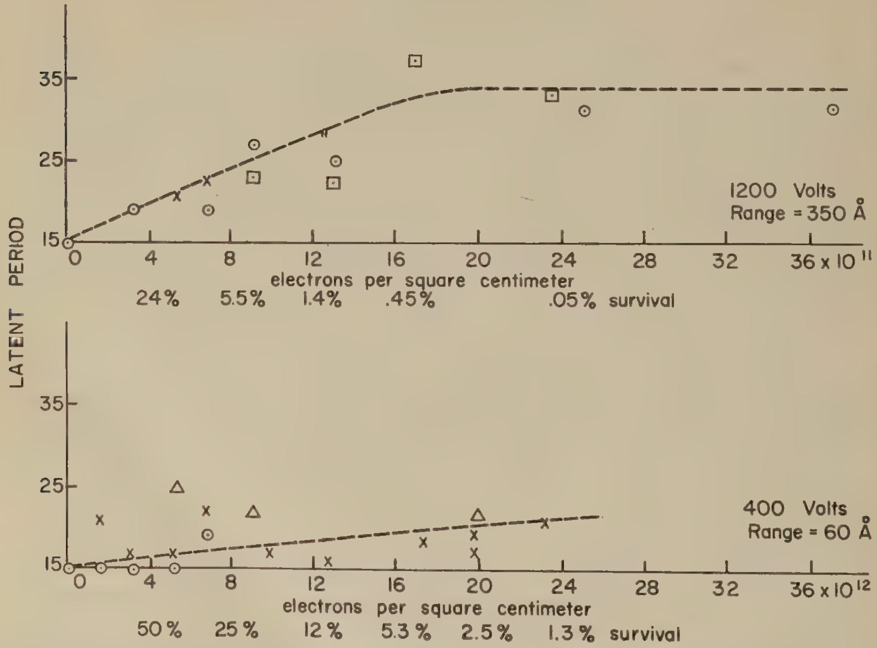

FIGURE 4. The effect of slow electrons on the latent period of T-1 bacteriophage: 400-volt electrons will be stopped by the protein coat, 1200 volt electrons will penetrate to the nucleic acid core.[8]

Samples cannot of course be irradiated in the wet state, not only because of the shielding effect of the water surrounding the organisms, but because of the possible transport of irradiation effects beyond the range of the particles by the diffusion of water radicals.

Ability to withstand vacuum is a very desirable property for target organisms, since the beam must be generated in a vacuum, and windows for a beam having the penetration suitable for work with microorganisms would be a severe technical problem.

Electron beams of suitable energies quickly build up large enough charges on insulators to deflect the beam. For this reason, all samples are irradiated on stainless steel cover slips, not on glass. The build-up of charge on an organism sufficient to deflect an electron beam is a possible complication. A rough calculation indicates that, if biological material has a resistivity the order of magnitude of celluloid ($\sim 10^{10}$ ohm-cm. units), the difficulty will not be too serious. An experimental check is to perform irradiations at different currents, the higher current giving more charging effects. Such a test, made during the irradiations of *Bacterium subtilis* spores as described below, showed no signs of charging effects.

Applications to Bacterial Spores

B. subtilis spores are roughly ellipsoidal in shape, with a major axis of 1.5 microns, and minor axes of 0.9 microns. The results of irradiating these spores with slow electrons[9, 10] are shown in FIGURE 5. Up to 200 volts, electrons have essentially no effect on the spores, indicating the presence of a tough, insensitive coat surrounding the spore. The range of such low voltage electrons is not known accurately, but is probably about 20 to 30 Å., which may be equated to the minimum coat thickness.

Between 300 and 900 volts, the inactivation curves are characterized by essentially zero initial slopes, indicating the presence of a requirement of hits from several particles to inactivate, and by a trail-off region, in which an increase in electron dosage produces no increase in inactivation. To understand the meaning of this latter phenomenon, a batch of spores was irradiated with 600-volt electrons with a dosage well out on the plateau, the surviving spores resuspended in water, and then relaid down for another irradiation. The second irradiation then produced exactly the same percentage inactivation as it would have if the spores had not previously been irradiated. The conclusion is clear that only a limited area of the spores is sensitive to 600-volt electrons. The electrons inactivate all those spores which have this sensitive region uppermost, but cannot affect those which have the region on the side of the spore away from the beam. Resuspending the spores rearranges them in a random way, so that the realigned spores have the same fraction with the sensitive regions accessible to the electrom beam as the original preparation.

At 900 volts and above, the inactivation curves are logarithmic, which is the sign of inactivation by a single particle. At this energy, it is clear that the electrom beam is penetrating the spore coat at any place, indicating a thickness of about 230 Å. Data have been taken to energies up to 3500 volts, cor-

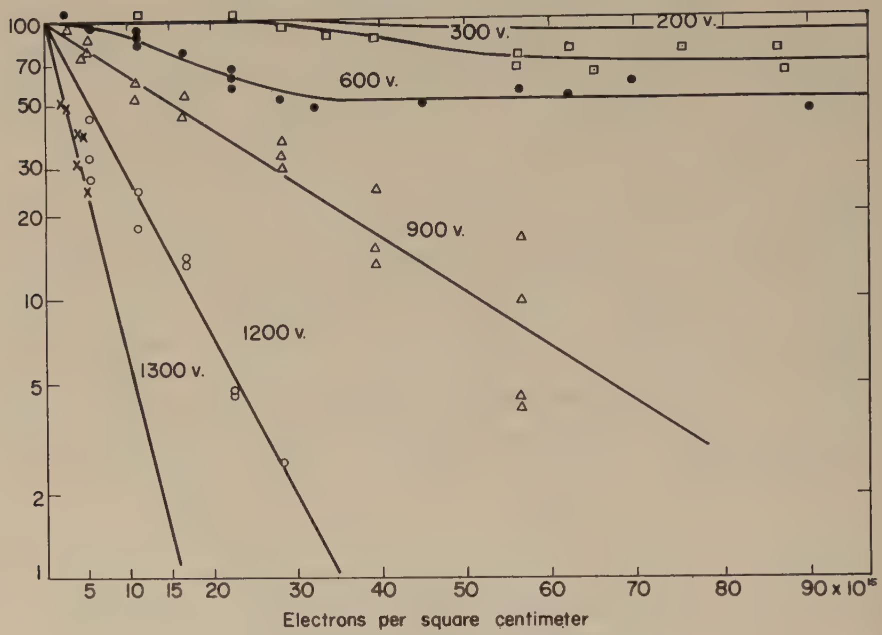

FIGURE 5. The effects of slow electrons on *B. subtilis* spores.[9, 10]

responding to a range of about 4000 Å., or almost half-way through the spore along a minor axis. The sensitivity of the spores increases rapidly with electron energy, but not more rapidly than would be expected, remembering that the scattering of the electron beam, as it passes through matter, is such that the number of electrons passing through the coat increases rapidly with beam energy.

From these data, the picture of a *B. subtilis* spore, shown in FIGURE 6a, was constructed. Essentially three regions are involved: a completely radiation-insensitive coat 230 Å. thick; a radioresistant region requiring multiple hits, lodged in the coat and coming within about 25 Å. of the surface at one point; and a radiosensitive core which either contains the genetic apparatus uniformly mixed in, or at least distributed in several widely-spaced discrete chunks.

It is interesting to compare this picture with the conclusions of Robinow shown in FIGURE 6b, based on electron micrographs of very thin (.3 to .4 micron) sections of *B. megatherium* spores.[11] In this case, Robinow's spore coat and cortical layer may be identified with the radioresistant outer coat. The matrix of the core is then to be correlated with the relatively radioresistant region which, in general, requires several hits to accomplish the inactivation of the spore. The chromatinic, or genetic material, well distributed throughout the core but apparently only rarely found in that portion of the core material which approaches the outer spore coat, is the very radiosensitive material, as might be expected. Altogether, the agreement is very satisfying.

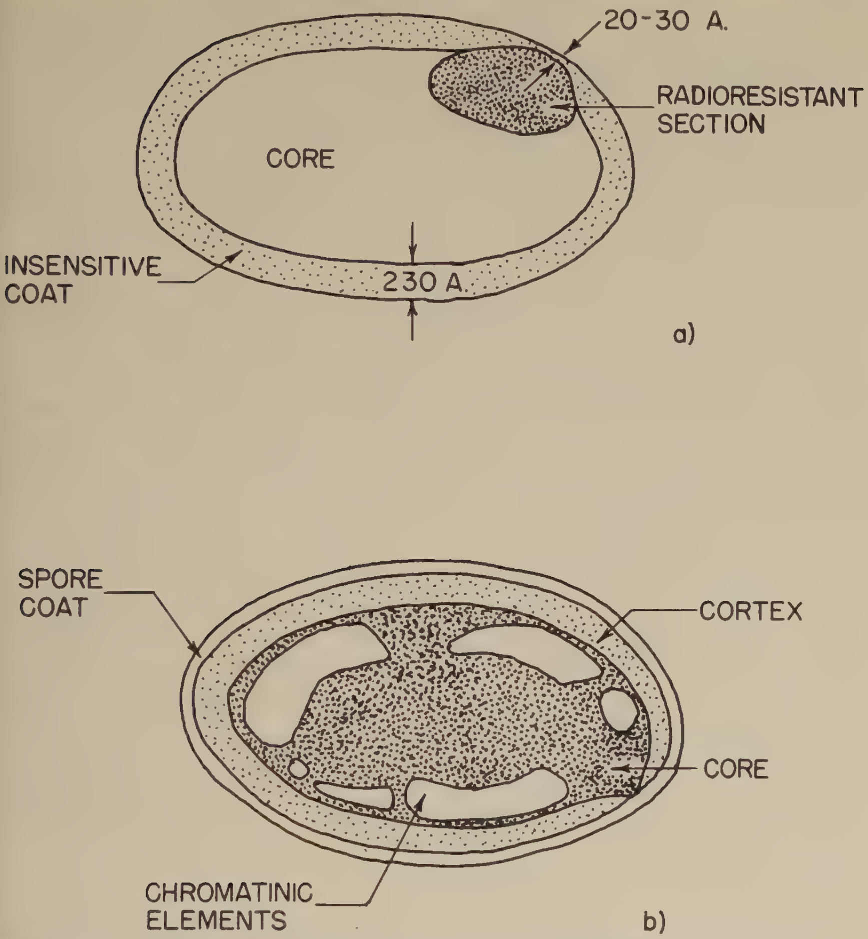

FIGURE 6. a) A picture of the structure of a spore constructed from the radiation data of FIGURE 5. b) The spore of *B. Megatherium* as given by Robinow.[11]

In conclusion, it can be said that some of the restrictions of the method are inherent, for example, the requirement that target materials be arranged in a single layer, but some of the other difficulties met so far are not intrinsic. Possible ambiguities in interpretation, due to the variation in radiation effect with depth caused by electron scattering, can be greatly reduced by using heavy particles such as protons. The increased mass of these particles will not only minimize scattering, but will also substantially reduce the possible deflection of incident particles from target organisms because of charging up.

The requirement that the targets be placed in vacuum will disappear when the systems under investigation have dimensons so great that the beams used to investigate them are high enough in energy to bring them easily through vacuum-tight windows. A possible case in point would be an investigation of the radiation sensitivity of the human skin with particle beams of range of the order of millimeters. The practical applicability of this suggestion lies in the possibility of doing cancer therapy with X rays of energy of the order of several million volts. Such X-ray beams produce their maximum ionization a distance of millimeters under the surface.[12] A knowledge of the relative radiation reaction of various components of the skin would enable a rational choice of the energy of X-ray machines to use for cancer therapy to minimize the undesirable skin reaction.

Summary

Charged particles, such as electrons or protons, have a finite range in tissue, the range depending on the energy of the charged particle. If the energy is made sufficiently low, the irradiation of an organism will be confined completely to the surface. Increasing the energy enables the charged particles to penetrate more deeply. By this method, it is possible to measure the thickness of a surrounding protective layer or skin, to ascertain the location of a biologically active unit (such as an antigenically active site) with respect to the surface, and to inactivate or otherwise interfere with a surface unit without directly affecting the underlying material.

Some recent applications of this method show its promise. On irradiating spores of *B. subtilis* with these electrons, it is found that the spore is covered by a radioresistant coat 230 Å. thick, except at one spot, where the coat is only about 25 Å. thick. There are definite indications that the genetic apparatus is not concentrated in a small volume within the spore, but is well dispersed throughout the central core. Applied to T-1 bacteriophage, the technique shows the existence of a radiation resistant coat 125 Å. thick surrounding the virus. Measurements of the effects on the latent period of T-1 of a low voltage beam confined to the protein coat and a higher voltage beam penetrating to the core show that the observed increase in latent period with irradiation is probably due to effects on the nucleic acid core of the virus.

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IRRADIATION OF PARTS OF INDIVIDUAL CELLS. III. EFFECTS OF CHROMOSOMAL AND EXTRACHROMOSOMAL IRRADIATION ON CHROMOSOME MOVEMENTS*

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The development of microbeams, both of protons[1] and of ultraviolet light,[2] ranging down to a few microns in diameter, has made possible the selective irradiation of small fractions of individual cells. This paper describes various effects on chromosome movements that result from irradiating chromosomes, parts of chromosomes, or other portions of cells in various stages of mitosis.

The procedure for proton microbeam irradiation has been detailed elsewhere.[1] The microbeam was approximately cylindrical but, because of scattering, some of the protons had trajectories outside the cylinder. For instance, when microaperture G-4 was used‡, 80 per cent of the protons striking a nuclear emulsion at the position of a cell under bombardment fell in a circle 2.5 μ in diameter, and 96 per cent within a circle 5 μ in diameter. When a slightly larger microaperture (G-5) was used, the corresponding diameters were about 3.5 and 6.5 μ respectively.

The ultraviolet microbeam[2] was a hollow cone (half angle 23°) that converged intensely into an approximately isodiametric volume about 7 μ across. The radiation was heterochromatic. The spectrum from a Hanovia quartz medium-high-pressure mercury arc was filtered through water to remove infrared. The ultraviolet spectrum was slightly modified by the 5-μ mica coverslip.

Fragments of heart from adult newts (*Triturus viridescens*) were cultured in a mixture of one volume of reconstituted chicken plasma (Difco TC) to six volumes of mammalian Tyrode solution and four of water. Cultures were incubated and used at 23° C. Because of geometrical requirements for proton microbeam irradiation,[1] only cells that were in contact with the cover slip were used. Such cells were flattened against the slip. Their nuclei were approximately ellipsoidal, the two horizontal axes being about 30 and 20 μ and the vertical about 10 μ. The normal diploid chromosome number was 24.

In each experiment, the cell was observed by medium-dark phase-contrast microscopy and was photographed, before and after irradiation, 15 times per minute on Eastman Super X 16-mm. film. Each figure shown in this paper was prepared by projecting a single 16-mm. frame on a screen and photographing it on Perutz Pergrano fine-grain film with a Leica camera. The actual area shown in each frame is about 60 × 40 μ.

Stages in division of nonirradiated cells are shown in FIGURES 1 and 2. FIGURE 1 is typical of the several hundred mitoses that we have photographed

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† National Science Foundation Fellow.

‡ See Figure 3 in Zurkle and Bloom.[1]

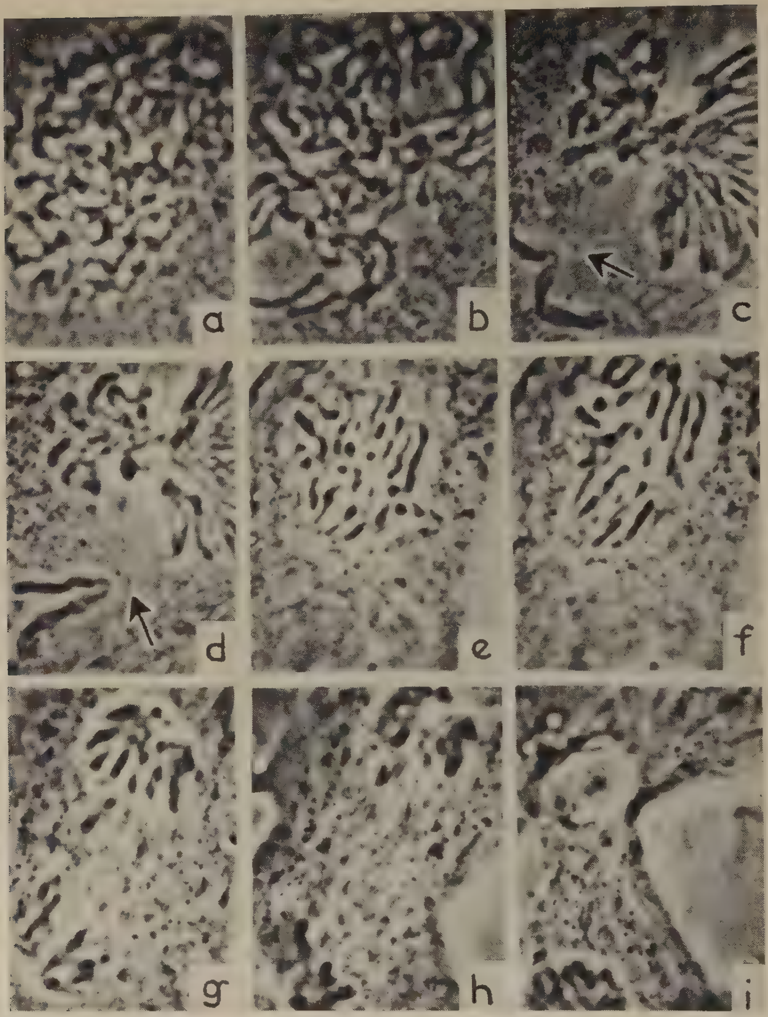

FIGURE 1. Mitosis in a nonirradiated cell (1-207) of adult newt heart in tissue culture. (a) Prophase. (b) Metakinesis. (c, d) Metaphase. One centrosome (arrow) and its centriole are shown. A "centrophilic" chromosome lies with its kinetochore adjacent to the centrosome. (e, f) Anaphase after centrophilic chromosome goes to equatorial plate). (g, h) Telophase. (i) Cytokinesis (constriction) has occurred. Daughter nuclei are reconstructing.

from prophase to reconstruction. Among these hundreds, in only one have we visualized the division of the centrosome as clearly as in FIGURE 2. In this particular cell, the centrosome and its daughters were clearly located by numerous, strongly attracted fat droplets (black in the photographs).

Effects on Metakinesis

The occurrence of centrophilic chromosomes (FIGURE 1c) in about half of the cells* makes it possible to irradiate a selected fraction of a chromosome and

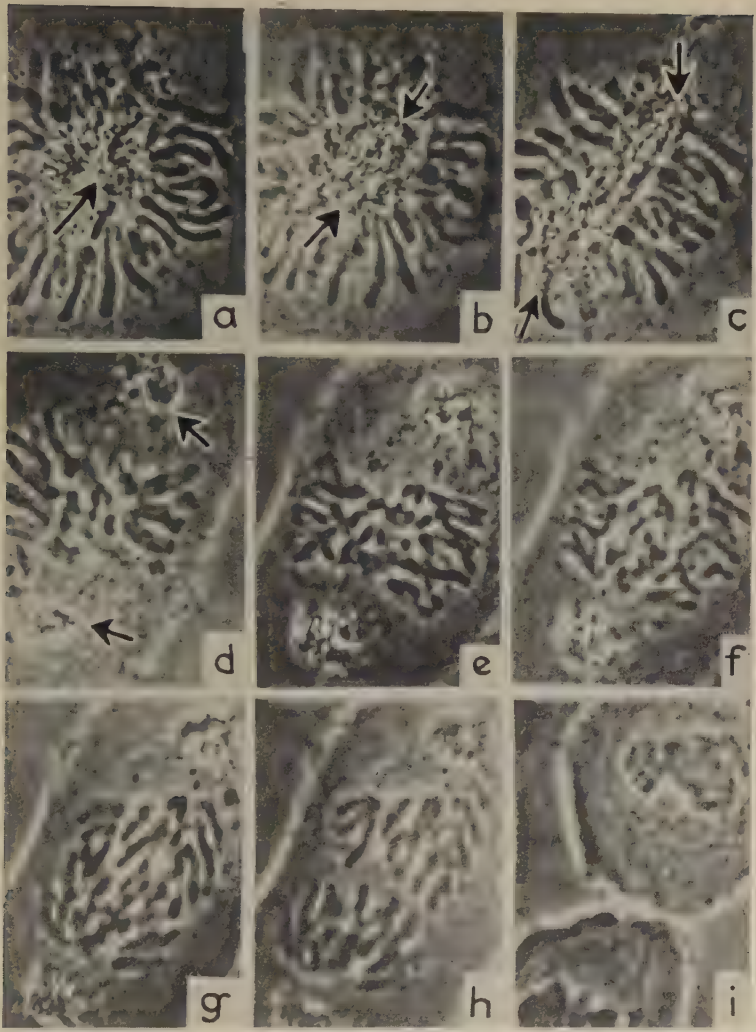

FIGURE 2. Mitosis in a nonirradiated cell (1-47), with special reference to division of the centrosome. Positions of the centrosome and its daughters are marked by the minute fat droplets that appear black and are attracted by the centrosome(s). (a) Prophase. Centrosome at center of group of fat droplets. (b) Daughter centrosomes starting to separate. (c) They have moved farther apart. Spindle outlined by fat droplets. (d, e) Metaphase. Fat droplets strongly attracted by centrosomes. (f, g) Early anaphase. (h) Late anaphase. Cytokinesis beginning. (i) Reconstruction of nuclei and completion of cytokinesis.

then observe the effect on its normal movement from centrosome region to equatorial plate.[2] Certain chromosomes were irradiated in a region containing the kinetochore; others in regions carefully selected not to contain it. A few were irradiated with protons; the behavior of one of these, in which the kineto-

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* These centrophilic chromosomes apparently occur at random, probably when the kinetochore of a metakinetic chromosome happens to pass sufficiently near a centrosome. The chromosome may remain centrophilic for 20 or 30 minutes, but regularly proceeds, kinetochore foremost, to the equatorial plate before anaphase. Some centrophils are V's, others J's. Their number per cell varies from zero to half a dozen, and they occur at one or both centrosomes. Accordingly, no given chromosome of the set is peculiarly centrophilic.

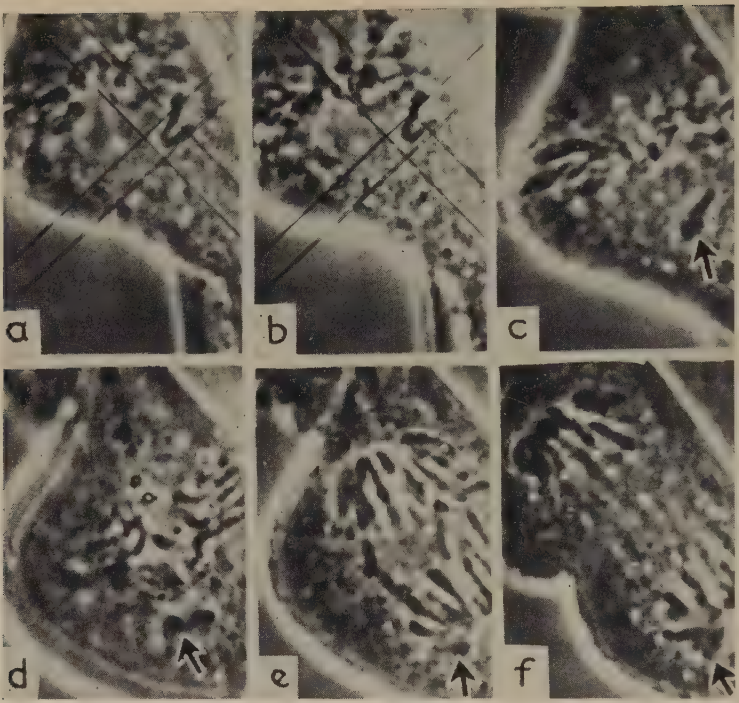

FIGURE 3. Behavior of a centrophilic chromosome bombarded in the kinetochore region with 90 protons (Cell 334, Microaperture G-4). (a) Before and (b) immediately after irradiation. Kinetochore at bend of chromosome. Bombarded area somewhat smaller than that enclosed by crosshairs. (c) Metaphase. Bombarded chromosome (arrow) drifting, aloof from equatorial plate. (d) Start of anaphase. Chromosome (arrow) still aloof. (e, f) Bombarded chromosome (arrow) is being pushed along by advancing anaphase chromosome group and later forms part of the resulting nucleus.

chore was exposed, is shown in FIGURE 3. The chromosome lost its ability to move in directed fashion; it drifted about until anaphase, and never joined the equatorial plate. Similar results were obtained with ultraviolet irradiation of the kinetochore region (FIGURE 4). Twenty chromosomes thus exposed for three minutes all failed to go to the plate. On the other hand, 15 chromosomes bombarded outside the kinetochore region all went to the plate before anaphase. Some were exposed three minutes, others six. One of the latter is shown in FIGURE 5.

The "paling" of irradiated chromosomes, illustrated in FIGURES 4 and 5, is due to a change in refractive index of the chromosomes. Since it is probably an indication of some basic change in submicroscopic chromosome structure, it is being investigated further. It progresses beyond the region of direct bombardment for 20 or 30 minutes after irradiation, so that the pale segment may ultimately become two or three times as long as the bombarded one. With ultraviolet light, paling results from exposures of about the same magnitude as those that inactivate the kinetochores. It also results from proton bombardment (not illustrated), but the required exposures are at least hundreds of times as great as those that inactivate kinetochores.

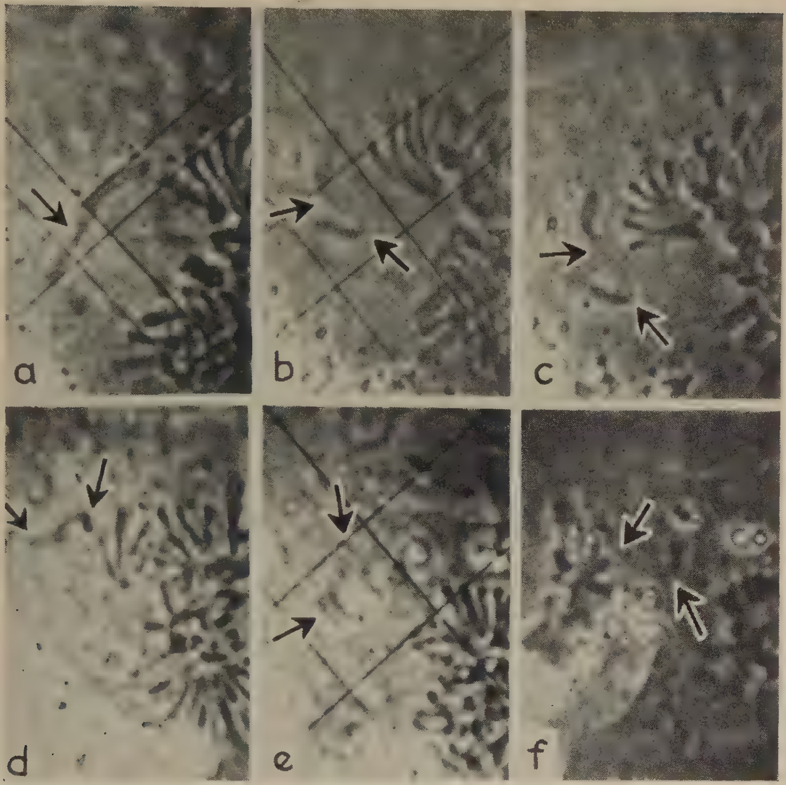

FIGURE 4. Behavior of a centrophilic chromosome irradiated for three minutes with ultraviolet light in a region containing the kinetochore (Cell 4-101). (a) Before irradiation. Kinetochore at arrow. (b) Immediately after irradiation. Irradiated portion pale. (c, d) Metaphase. Bombarded chromosome (arrows) aloof from equatorial plate. (e) Early anaphase. Chromosome (arrows) still aloof. (f) Telophase. Chromosome (pale segment between arrows) has been bypassed by the anaphase chromosome groups and is squeezed into left daughter cell by the cell constriction.

Effects on Anaphase Movement

Effects of chromosomal irradiation. Microbeam irradiation of prophase chromosomes with a few dozen protons is very effective in producing chromosome stickiness, akinetic chromosomes, and chromosome fragments (FIGURE 6). Irradiation of metaphase chromosomes is approximately as effective in producing stickiness, but very rarely produces akinetic chromosomes or fragments.

An interesting application of production of stickiness is the formation of anaphase hinges by suitable exposure of one side of the metaphase chromosome configuration (FIGURES 7 and 8). Sticky bridges are produced only in the irradiated chromosomes. The nonirradiated ones attempt a normal anaphase movement and swing *as a group* around the sticky ones as a hinge. Hinges up to 90° have been produced by some dozens or hundreds of protons.[1] With a few thousand protons, we have produced several that exceed 90° (FIGURE 7),*

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* A hinge angle is the one formed by the tangents drawn to the two advancing chromosome fronts.

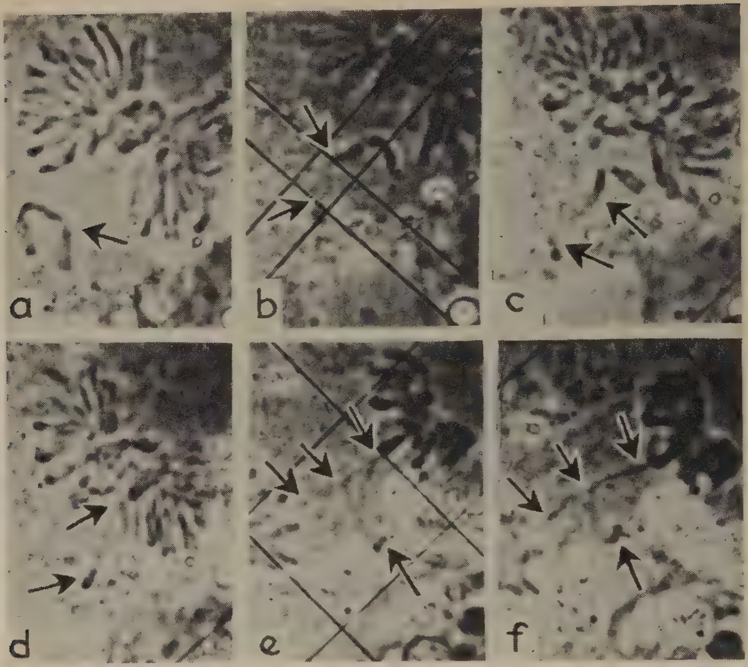

FIGURE 5. Behavior of a centrophilic chromosome irradiated for six minutes with ultraviolet light in a region not containing the kinetochore (Cell 4-97). (a) Metaphase, before irradiation. Kinetochore at arrow. (b) Immediately after irradiation. Bombarded region pale (between arrows). (c, d) Chromosome (pale region between arrows) moving to equatorial plate. (e, f) Telophase. Irradiated chromosome has undergone anaphase movement with the rest, but the chromatids are stuck in the pale, irradiated portion and cannot separate, a T-shaped bridge (arrows) resulting.

and the same result has been obtained with suitable ultraviolet microbeam exposures (FIGURE 8). Since this hinge phenomenon probably can throw some light on the natures(s) of the forces involved in late anaphase, it will be further investigated.

Effects of extrachromosomal irradiation. In contrast to the marked effectiveness of a few dozen protons delivered to small chromosomal regions, relatively huge numbers were ineffective when delivered to similarly small extrachromosomal regions. Thousands were delivered to ends of spindles, and hundreds of thousands to cytoplasm. No effects could be seen at the irradiation sites or elsewhere. In some cells, a sticky chromosome or two were observed. Such chromosomes are ascribable to the few dozen protons that were scattered into the chromosomes, when such large numbers were aimed at extrachromosomal targets.

In contrast to the protons, heterochromatic ultraviolet radiation produced very striking effects when extrachromosomal regions were given exposures of the same order as those which, given to chromosomal regions, produced stickiness, paling, and inactivation of kinetochores. When one or both ends of a spindle and the adjacent cytoplasm were irradiated, the typical effects were as shown in FIGURE 9. Within 20 minutes after exposure, the spindle disappeared,

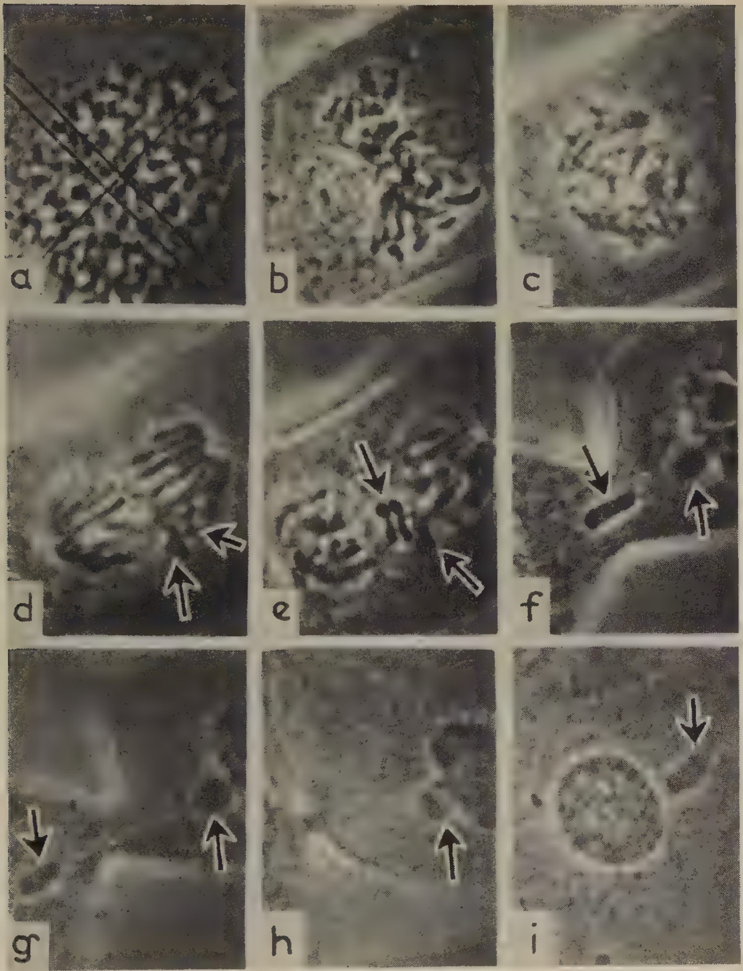

FIGURE 6. Chromosome aberrations produced by 30 protons to a small fraction of a prophase chromosome group (Cell 349, microaperture G-4). (a) Prophase. Bombardment at crosshairs. (b) Metaphase. (c) Early anaphase. (d) Late anaphase. Bombarded chromosomes at arrows. (e) Telophase. Bombarded chromosomes and/or fragments left behind the normal anaphase chromosomes. (f) One chromosome (or large fragment) drifting (left arrow). Others (right arrow) attached by sticky bridges to one of main chromosome groups. (g) Drifting chromosome squeezed into left daughter cell by cytoplasmic constriction. (h) Sticky chromosomes form large lobe on right daughter nucleus. (i) Drifting chromosome forms separate accessory nucleus in left daughter cell.

and the chromosomes lost their characteristic metaphase configuration (compare FIGURE 9c with 9a). Then the chromosomes slowly became arranged in a "quasirosette" configuration,* with all or most of the kinetochores pointing toward a small central region (FIGURE 9d). This stage ("deranged metaphase") usually lasted from two to four hours. Then, in at least half and probably more of the cells, two groups of chromosomes moved apart (FIGURES 9e 9h). Each

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* This configuration is reminiscent of some effects of colchicine produced under certain conditions, *e.g.*, in the grasshopper neuroblast as described by Gaulden and Carlson.[8]

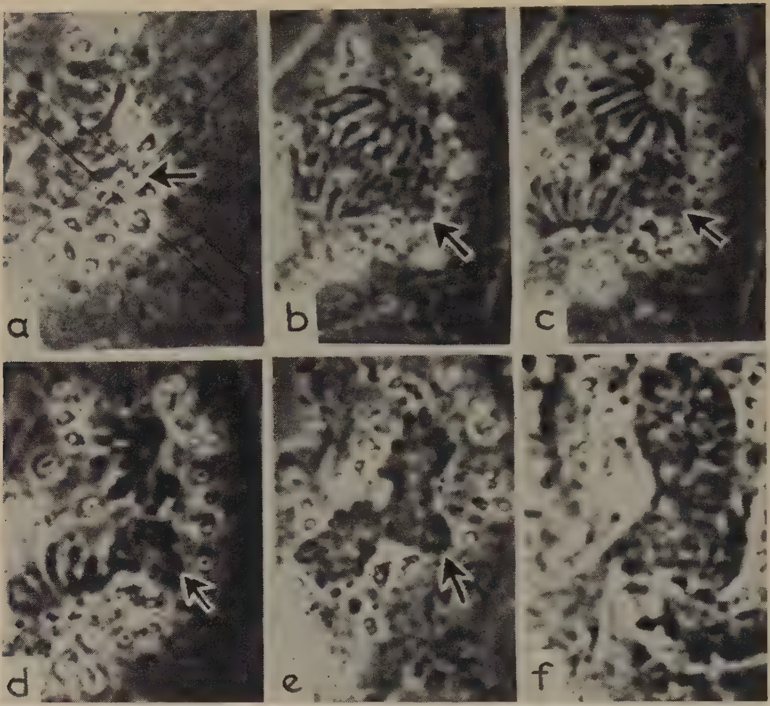

FIGURE 7. Anaphase hinge produced by 2400 protons localized to one side of metaphase chromosome configuration (Cell 363, Microaperture G-5). (a) Chromosomes irradiated in area indicated by crosshairs and arrow. (b) Anaphase. Irradiated chromosomes granular (arrow). (c) Hinge about 60°. (d) Hinge about 130°. (e, f) Reconstruction and cytoplasmic constriction.

of these groups was essentially a quasirosette, widely different in appearance from a true anaphase group (FIGURES 1 and 2). In some cells, approximate chromosome counts could be made. In all these cases, the total number in the two separating groups was 24, which is the number normally in each group of a true anaphase. The groups were not always 12 and 12; combinations such as 11 and 13, 10 and 14, *etc.* frequently occurred. Evidently, in this abnormal process involving no visible spindle, groups of whole chromosomes instead of half-chromosomes (chromatids) were separating. This process we have termed "false anaphase."*

To destroy the spindle and induce the deranged metaphase and false anaphase, it is not necessary to include part of the spindle in the irradiated region. A slightly greater exposure of an equal volume of cytoplasm produced the same effects. Since, in the spindle irradiations, cytoplasm also was exposed,

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* Qualitatively, false anaphase can be partially accounted for in terms of normal behavior of certain cell parts. It is possible that the ultraviolet irradiations described are quite selective and destroy only the spindle, leaving other cell parts essentially normal. The two centrosomes, with the spindle gone, may come close together. Then, since the chromosomes in these cells are known to be naturally quite centrophilic (FIGURE 1), and since there is no spindle to influence them, it is easy to see how the kinetochores might approach the two adjacent centrosomes, thus forming the observed quasirosette. Now, centrosomes normally move apart in the early stages of mitosis. If this ability is retained by the centrosomes assumed to be at the middle of the quasirosette, then their separation, each accompanied by approximately half of the centrophilic chromosomes, would account for the observed false anaphase movement.

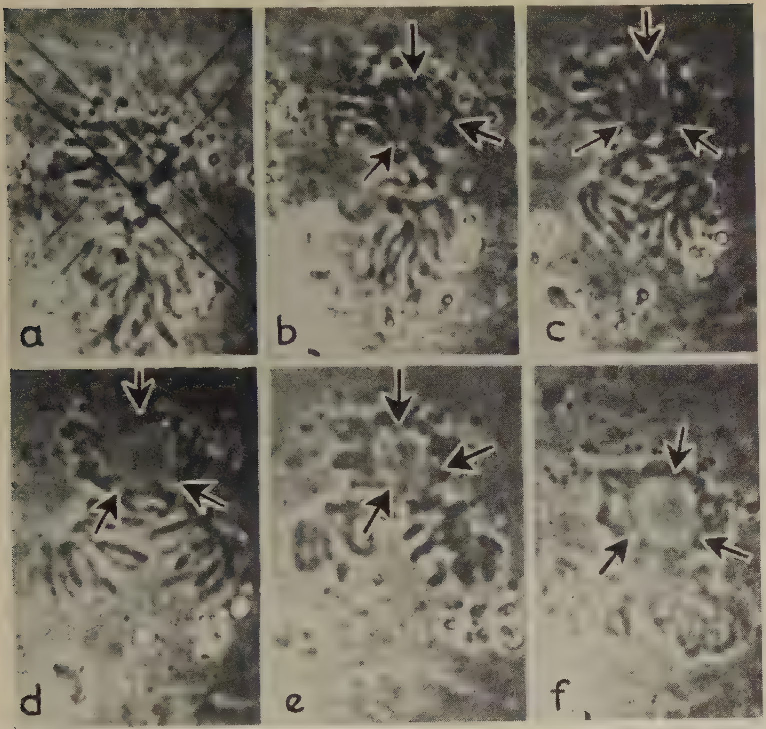

FIGURE 8. Anaphase hinge produced by three minutes of ultraviolet microbeam irradiation to one side of metaphase chromosome group (Cell 4-216). (a) Area bombarded indicated by crosshairs. (b) Immediately after irradiation. Chromosomes pale at bombardment site (arrows). (c) Anaphase starting. (d) Hinge about 95°. (e) Hinge about 105°. (f, g) Reconstruction of single large nucleus. Paling persists (arrows).

these effects are probably due entirely to absorption of ultraviolet by some cytoplasmic component. As might be expected, they could also be produced by a suitable total-cell exposure (FIGURE 10) although, in this case, much chromosome stickiness also occurred, since the chromosomes as well as the cytoplasm were irradiated.

After false anaphase, the cytoplasm constricted between the two groups of chromosomes, and the latter reconstructed into normal-appearing nuclei (FIGURE 10), very much as occurs after true anaphase.

Summary

Selected parts of mitotic cells in cultures of newt heart were irradiated with proton microbeams 2.5 to 3.5 μ in diameter or with an ultraviolet microbeam 7 μ in diameter.

If, during metakinesis, the kinetochore region of a chromosome was irradiated with either protons or ultraviolet, the chromosome lost all capacity for directed movement and simply drifted. No such effect followed irradiation of any other equal portion of a chromosome, even though the exposure was doubled. Drifting (akinetic) chromosomes were also readily produced by proton irradiation

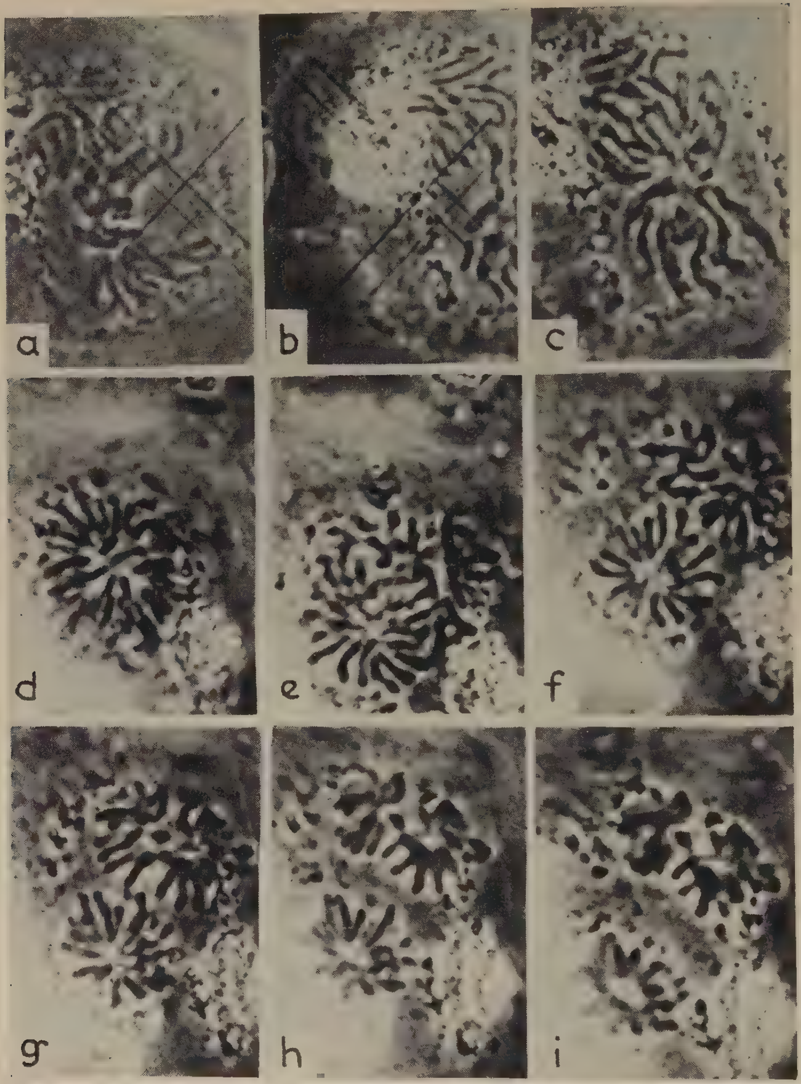

FIGURE 9. Deranged metaphase and false anaphase produced by 1.5 minutes of ultraviolet microbeam irradiation to each end of the spindle and the circumjacent cytoplasm (Cell 4-142). (a) Before irradiation. One site of bombardment indicated. (b) Immediately after irradiation. The other site of bombardment indicated. (c) Twenty minutes after bombardment. Spindle no longer visible. Chromosome configuration deranged. (d) One to four hours after irradiation. "Quasirosette" arrangement of chromosomes: kinetochores pointed toward a common point. (e, f, g) Two groups of entire chromosomes (not chromatids) move apart. (h) Reconstruction of nuclei beginning. (i) Reconstruction progressing. Constriction beginning.

of a few per cent of the prophase chromosome configuration. Whenever a drifting chromosome or chromosome fragment was formed, the cytoplasmic constriction (cytokinesis) squeezed it into one of the daughter cells, and it either formed an accessory nucleus or became a part (usually a lobe) of the main daughter nucleus.

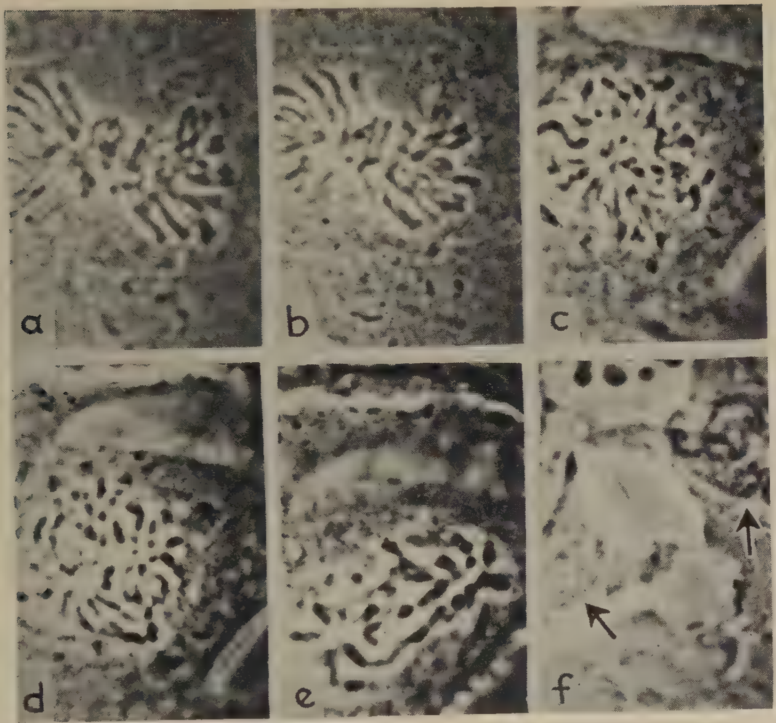

FIGURE 10. Deranged metaphase and false anaphase produced by 45 seconds of total-cell irradiation 2.5 cm. from a 15-watt General Electric germicidal lamp (cell 3-21). (a) Before irradiation. (b) A few minutes after irradiation. Spindle disappearing and chromosome configuration becoming deranged. (c) "Quasirosette" arrangement of chromosomes. (d, e) Two groups of chromosomes move apart. (f) Reconstruction of nuclei (arrows) and cytoplasmic constriction. Sticky bridge.

When one side of the metaphase chromosome configuration was suitably exposed to protons or ultraviolet, sticky anaphase bridges formed on the irradiated side. The tension in such a bridge, along with the normal anaphase forces, produced a "hinge," which in some cases swung as much as 135°.

Irradiation of portions of the spindle or of the cytoplasm with a 3.5-μ proton beam produced no observable abnormalities of any sort even though the exposures were respectively 100 and 10,000 times as great as those that produced chromosome stickiness (bridges) and drifting chromosomes. However, with the ultraviolet microbeam and exposures only a few times as great as those producing stickiness, irradiation in the cytoplasm regularly caused the spindle to disappear and the metaphase chromosomes to lose their characteristic configuration and lie haphazardly. After suitable exposures, this "deranged metaphase" was followed by a "false anaphase," in which two groups of whole chromosomes instead of half-chromosomes (chromatids) separated.

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THE EFFECTS OF X-RAYS, 2-MEV ELECTRONS, THERMAL NEUTRONS, AND FAST NEUTRONS ON DORMANT SEEDS OF BARLEY*

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Introduction

It is of interest that 26 years after L. J. Stadler (1928) demonstrated that X rays could be used to induce mutations in plants, barley (*Hordeum vulgare*), the species which he used still figures prominently in radiobiological investigations. One obvious reason for the continued use of barley in such studies is that this plant affords the biologist the opportunity to use several criteria for measuring radiation effectiveness following any given treatment. For example, after seeds have been irradiated, the effects of the treatment can be observed by determining: (1) the frequency of chromosomal aberrations, at the first cycle of cell divisions; (2) the growth rate inhibition and total growth of seedling plants; (3) the frequency of monocentric reciprocal interchanges and other chromosomal anomalies that persist to the meiotic divisions; (4) induced sterility, as evidenced by seed set of the irradiated generation; and (5) the frequency of seedling and mature plant mutations in the X_2 generation (the irradiated generation is designated as the X_1, the following generation as the X_2).

Proper utilization of the various criteria that can be used to measure radiation effectiveness permits one to determine whether two or more observable events resulting from a particular treatment are mutually exclusive or have varying degrees of interdependence. That this approach to radiobiological problems has actual as well as potential value in elucidating the complexities of biological disturbances caused by ionizing radiations will be illustrated in this report.

Recent studies conducted both in Sweden and in this country (MacKey, 1951, 1952; Caldecott *et al.*, 1952) have indicated that radiations with high and low specific ionizations differentially induce biological events resulting in death and/or in chromosomal aberrations induced in treated seeds of barley. If detectable differences do indeed exist, it was reasoned that much data of biological significance could be gleaned from a study relating ion density to as many of the above criteria as possible. This report describes research undertaken with this objective.

Seed Source and Cytogenetic Methodology

Seeds of Himalaya barley (*Hordeum vulgare*) were used for the entire study. This strain is hull-less and has a high degree of self-fertility. Self-fertility insures genetic purity, and this characteristic was further exploited by using

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* A portion of the research reported in this paper was carried out at the Brookhaven National Laboratory under the auspices of the United States Atomic Energy Commission.

seeds derived from a single plant selection made from a bulked population in 1947.

The excellent staining characteristics of the chromosomes in the Himalaya strain render it particularly suitable for cytogenetic analyses. The haploid chromosome number is seven, and four of the chromosomes can be easily identified in well-prepared slides.

When refrigerated, specimens for cytogenetic studies can be suitably fixed and stored in Carnoy's Solution (six parts of ethyl alcohol; three parts of chloroform; one part of glacial acetic acid) for as long as two years. Excellent permanent and temporary smears of meitoic and mitotic division figures can be obtained with propriono and aceto carmine or orcein. The carmine dyes are preferable, and were used throughout the present study.

Prior to irradiation, the seeds were stored in a desiccator over dry calcium chloride until they had reached a constant weight. After irradiation, which involved exposure to X rays, 2-mev electrons, thermal neutrons, or fast neutrons, the seeds were handled in one of the following ways: (1) some seeds were placed on blotters in petri dishes and allowed to grow for seven days, after which height measurements were taken; (2) other seeds were grown under carefully controlled greenhouse conditions to obtain information pertaining to survival as a function of dose; (3) other samples were grown under field conditions. From the material grown in the field, pollen mother-cell specimens were obtained at the time of meiosis and used for cytogenetic studies. The same plants were permitted to mature, and the seeds harvested were used for seedling mutation studies. In addition, counts of survival to maturity were made.

Radiation Sources

The X rays used in this study were generated with a G. E. Maxitron Facility operated at 250 kvp. and 30 ma, and were filtered through 1 mm. of aluminum. The seeds, which were in a Pyrex petri dish, received about 800 r. per minute at their surface as measured with a Victoreen Integron.

The 2-mev electrons were obtained from a Van de Graaff Electrostatic Generator. The seeds were exposed to the electron beam while in a vacuum bombardment chamber designed by Doctor D. J. Fluke, who also supervised the dosimetry. The machine was operated so that the seeds received approximately 13,200 roentgen equivalent physical (rep.) every seven seconds.

The thermal neutron irradiations were conducted in the thermal column of the Brookhaven National Laboratory's Nuclear Reactor. Seeds were exposed to the particles while they were in a single layer in a lucite container.

It should be pointed out that it is a characteristic of thermal neutrons that, when they come close to atomic nuclei, they are attracted by short-range nuclear forces and may be captured by the nucleus. When capture takes place, the thermal neutron contributes very little or no kinetic energy to the atom, but it does contribute its binding energy. For most elements found in biological materials, the compound nucleus formed from thermal neutron capture releases this excess energy in the form of gamma radiation. Two notable exceptions are nitrogen, which emits a 0.6-mev proton and boron which emits a 2.4-mev alpha particle. Through calculations relating the abundance of different elemental constituents in biological material, and the capture cross section of the elements for thermal neutrons, it is possible to determine the amount of ionization attributable to a particular species of radiation (see Conger and Giles, 1950). Using calculations similar to those of Conger and Giles, it has been shown that over 90 per cent of the ionization produced in barley seeds from thermal neutron capture is from protons from the reaction ${}_7N^{14} + {}_0n^1 \rightarrow {}_6C^{14} + {}_1H^1 + 0.6$-mev., and that about 8 per cent is from alpha particles from the reaction ${}_5B^{10} + {}_0n^1 \rightarrow {}_3Li^7 + {}_2He^4 + 2.4$-mev.

It is significant that the gamma contamination in the Brookhaven Thermal Column is less than 150 r. per hour, which rendered it of little importance in these studies. In a similar way, the fast neutron contamination was relatively insignificant, as indicated by a cadmium ratio of about 5000:1.

The flux, or number of thermal neutrons in the thermal column, was determined by exposing gold foils during each treatment and relating the radioactivity induced in the foils to the number of neutrons present.

The fast neutrons used in the investigation were obtained from the fission of U^{235}. This was achieved by putting a U^{235} plate, covered with aluminum, in the thermal column of the reactor. Placed directly on top of the U^{235} plate was one inch of bismuth, which was also in an aluminum container. The bismuth was used to attenuate the gamma radiation which results from the fission process.

Seeds were exposed to the fast neutrons while in a lucite chamber in direct contact with the aluminum covered bismuth. No precise measurement of the flux of fast neutrons is available, but preliminary calculations made by Doctor H. J. Curtis indicate that, with the U^{235} plate in position, there are about equal numbers of fast and slow neutrons in the column. The fast neutron flux was calculated to be about $3.5 \times 10^8\ N_f/\text{cm.}^2/\text{sec.}$ when the pile is operated at 24 megawatts with a "standard" rod pattern. All fast neutron experiments reported here were conducted at this pile power, using the "standard" rod pattern.

A biological calibration with seeds of barley (FIGURE 5) showed that four hours' exposure to slow neutrons at a flux of $7.0 \times 10^8\ N_{th}/\text{cm.}^2/\text{sec}$ inhibited seedling growth to about the same degree as 25 minutes' exposure when the U^{235} plate was in position. From this finding it was calculated that the thermal neutron contamination recorded during the fast neutron exposure accounted for about 5 per cent of the observed biological effect. The gamma contamination was of even less significance. For this reason, no effort was made to subtract the effects due to contamination from the fast neutron data that are presented.

Fast neutrons principally effect ionization in biological materials by setting hydrogen nuclei (protons) into motion through collision. The fast neutrons from U^{235} are emitted in a spectrum with an average energy of about one mev and with a relatively minor percentage of the spectrum above four mev. From this it follows that the protons emitted in biological materials from hydrogen recoils will have a rather wide distribution of energies.

In this presentation, we shall consider that X rays and 2-mev electrons represent radiations with a relatively low specific ionization. It is recognized that, as we use them, a distribution of ion densities occurs with both of these radiation sources. However, a relatively large fraction of the energy loss occurs at a low specific ionization. This is not to impute the possible significance of the more densely ionizing parts of the energy-loss spectrum, such as that occurring in electron "tails."

We shall consider that thermal and fast neutrons represent sources of radiation yielding high specific ionization, organized along the tracks of the protons and alpha particles. In turn, this consideration is not to neglect the more sparsely ionizing protons produced by the faster neutrons in the fast neutron spectrum.

This study, then, is concerned with comparing the biological effects of radiations which have, for the most part, a high specific ionization with radiations which have a low specific ionization.

Effects of Ionizing Radiations on Seedling Growth

Earlier studies have shown that when barley seeds, as well as seeds of other species, are subjected to a dose of X rays sufficient to reduce seedling growth materially, the seeds are not uniformly injured, and the distribution of seedling heights about the mean is skewed. It was accordingly a point of some interest when, in our studies, we found that seedlings from seeds treated with any dose of thermal neutrons, irrespective of the reduction in seedling growth which resulted from the treatment, were uniformly injured, giving a normal distribution of heights about the mean.

All efforts to explain the lack of uniformity with X rays, either on the geometrical distribution of the X-ray beam and/or of the seed, failed. It was accordingly decided that this lack of uniformity must result from the physical nature of the radiation. From this inference, and from the fact that thermal neutrons have a much higher specific ionization than X rays, it seemed reasonable to assume that the difference may result from the spatial distribution of ion pairs in the irradiated material. In order to check this reasoning, and to preclude the possibility that the observed phenomena resulted from some experimental idiosyncracy, it was decided that the results should be compared with two different sources of radiation which had specific ionizations comparable to X rays and thermal neutrons.

Two mev electrons and fast neutrons were selected for this purpose. Dormant seeds were subjected to both these radiations, as well as to X rays and thermal neutrons, and analyzed to determine the distribution of seedling heights from a number of doses.

Frequency distributions of seedling heights, from experiments using all sources of radiation, are presented in FIGURES 1 to 4. From these figures, it is apparent that an irregular distribution of seedling heights was obtained with 2-mev electrons similar to that obtained from X radiation, and that a normal distribution of seedling heights was obtained with fast neutrons similar to that found with slow neutrons. To elucidate further the difference between the two types of radiations, doses of each radiation which caused a comparable

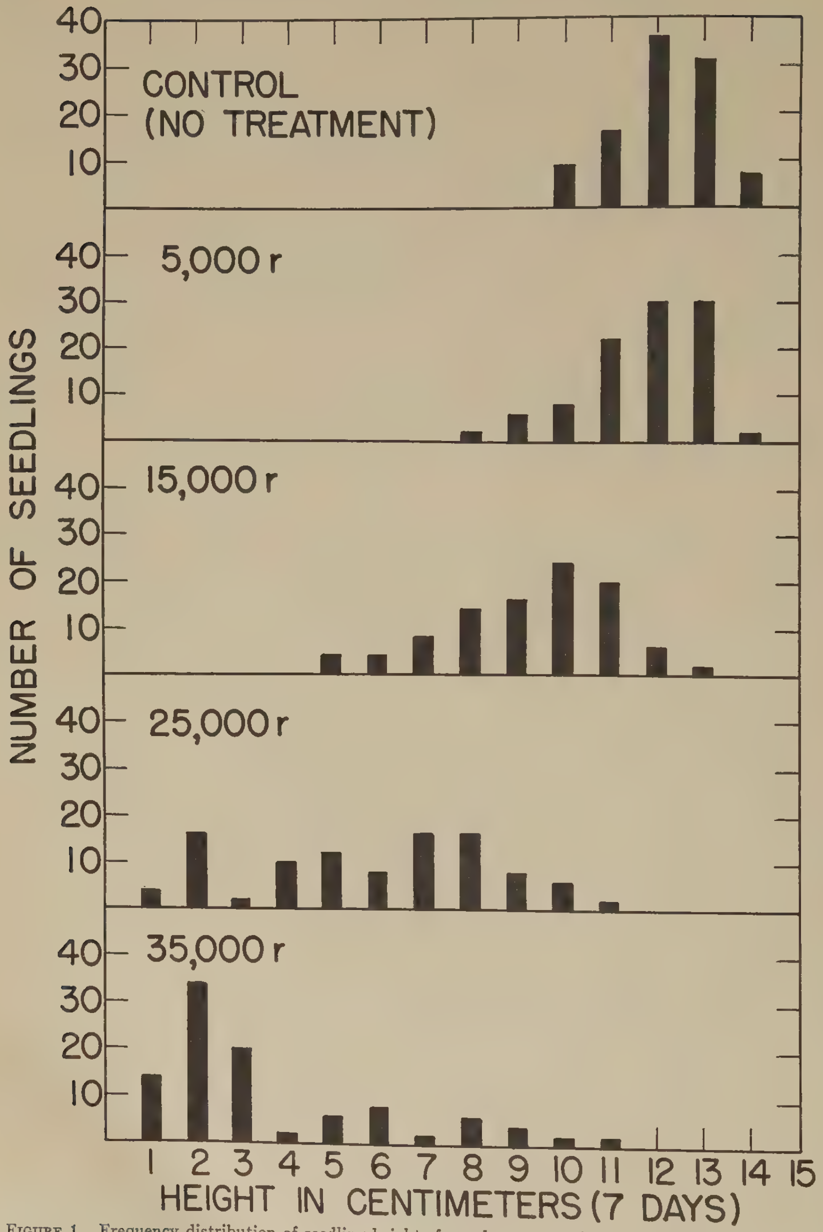


FIGURE 1. Frequency distribution of seedling heights from dormant seeds of barley subjected to different doses of X rays.

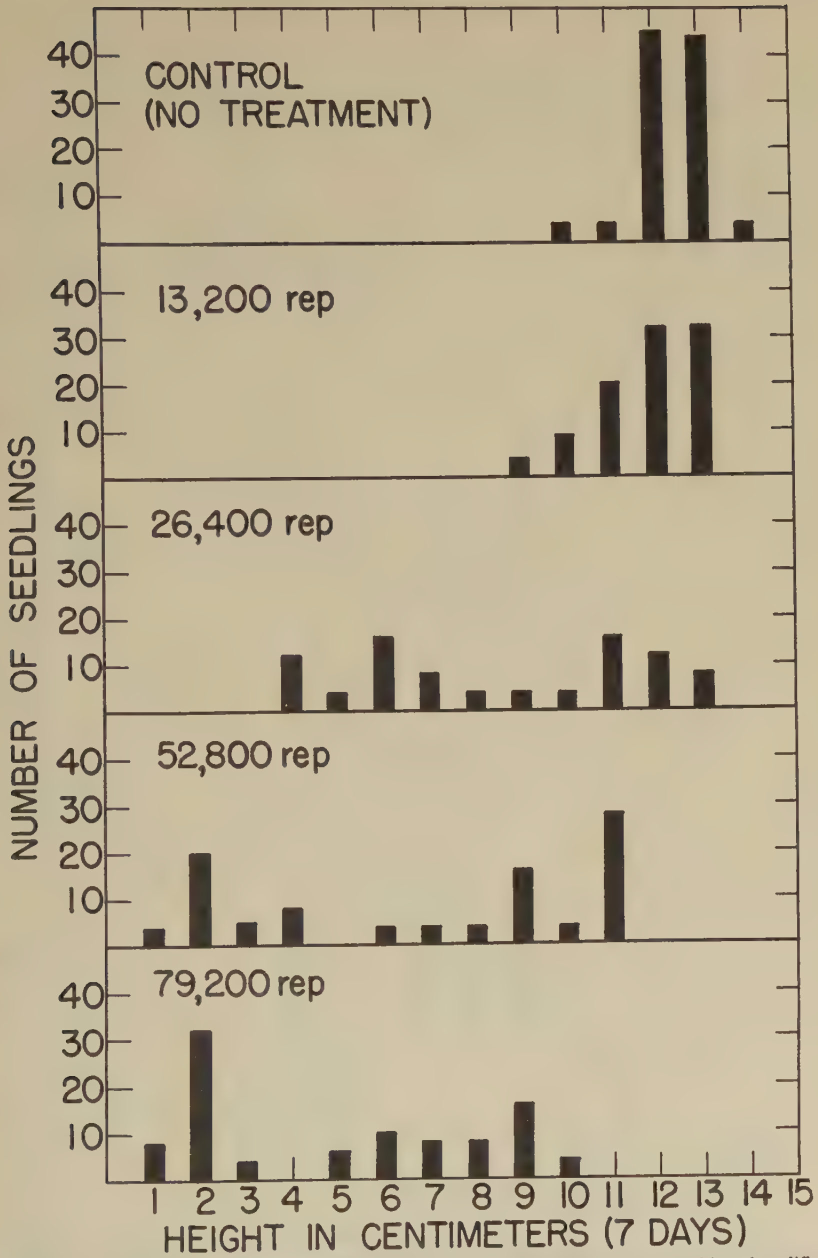


Figure 2. Frequency distribution of seedling heights from dormant seeds of barley subjected to different doses of 2-mev electrons.

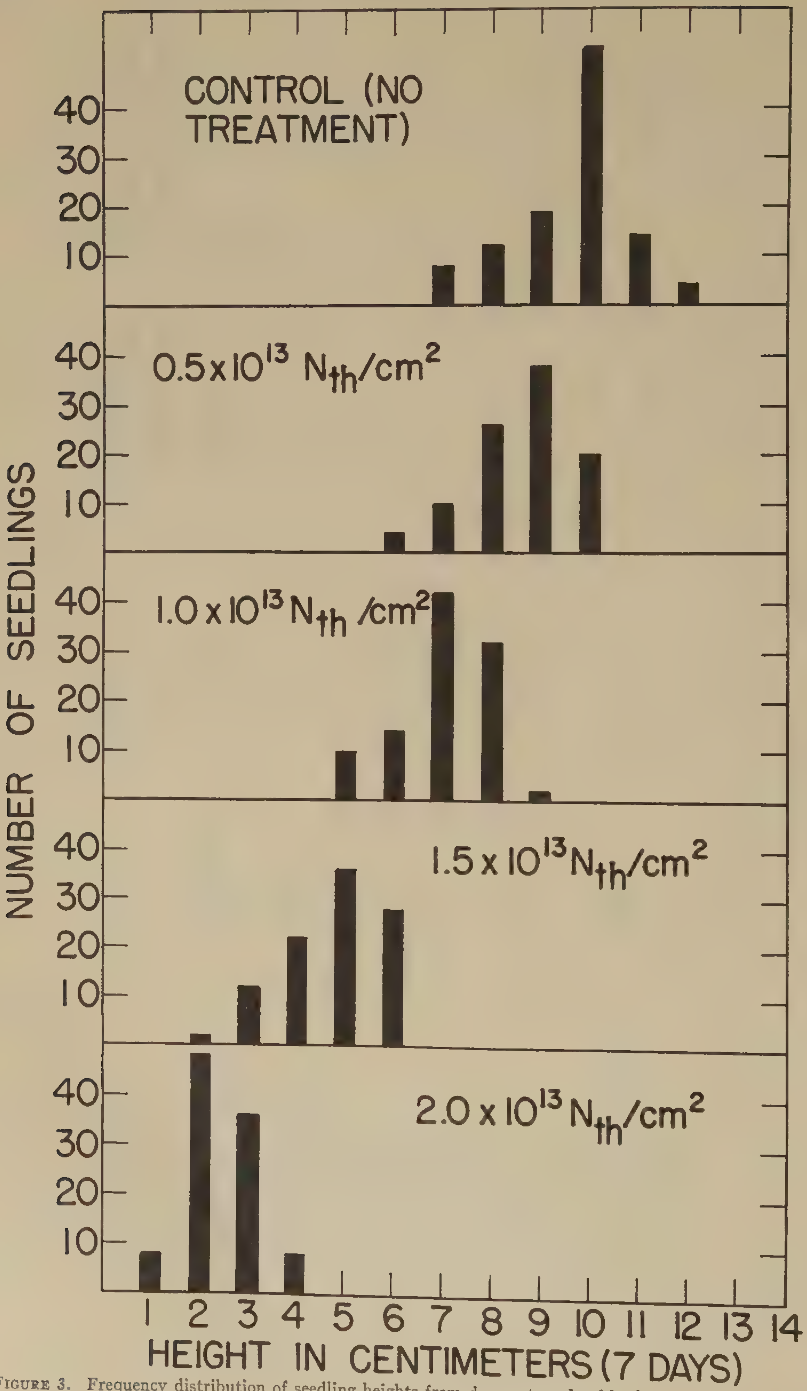


FIGURE 3. Frequency distribution of seedling heights from dormant seeds of barley subjected to different doses of thermal neutrons.

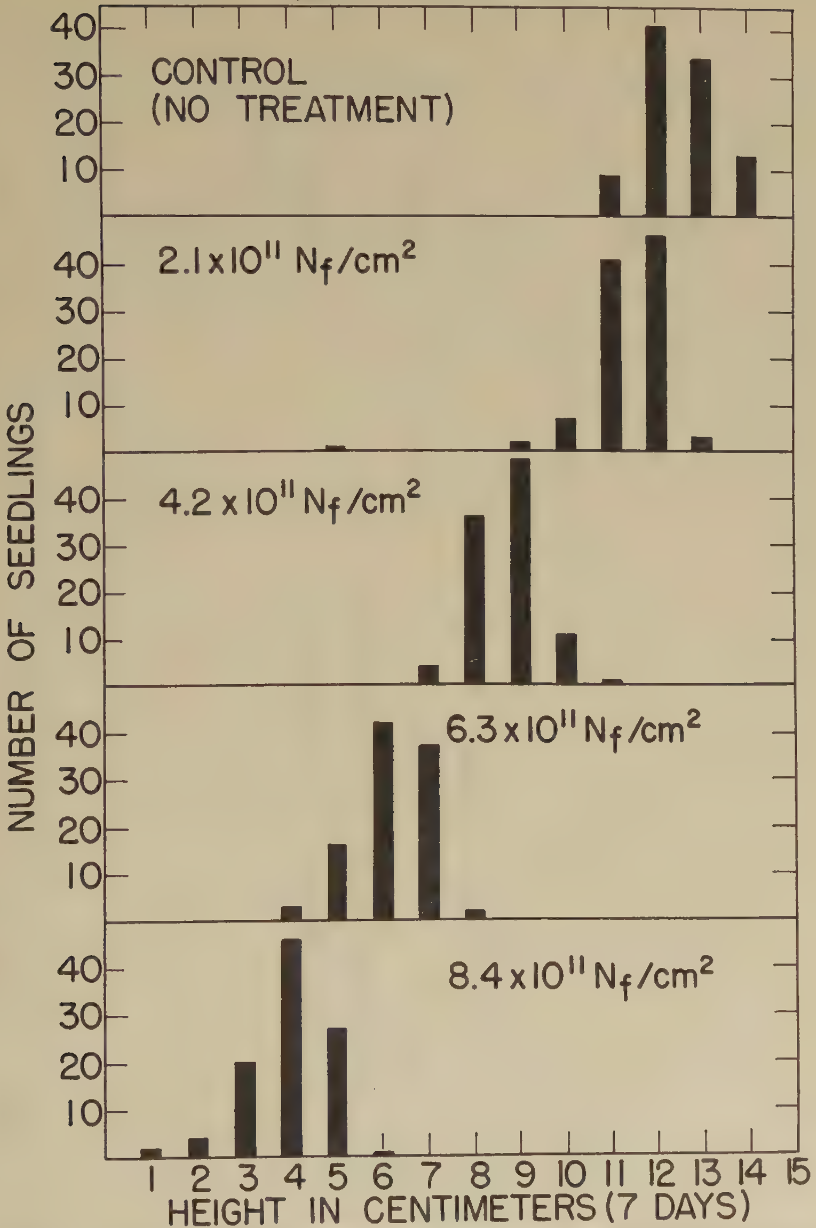


FIGURE 4. Frequency distribution of seedling heights from dormant seeds of barley subjected to different doses of fast neutrons from fission of U^{235}.

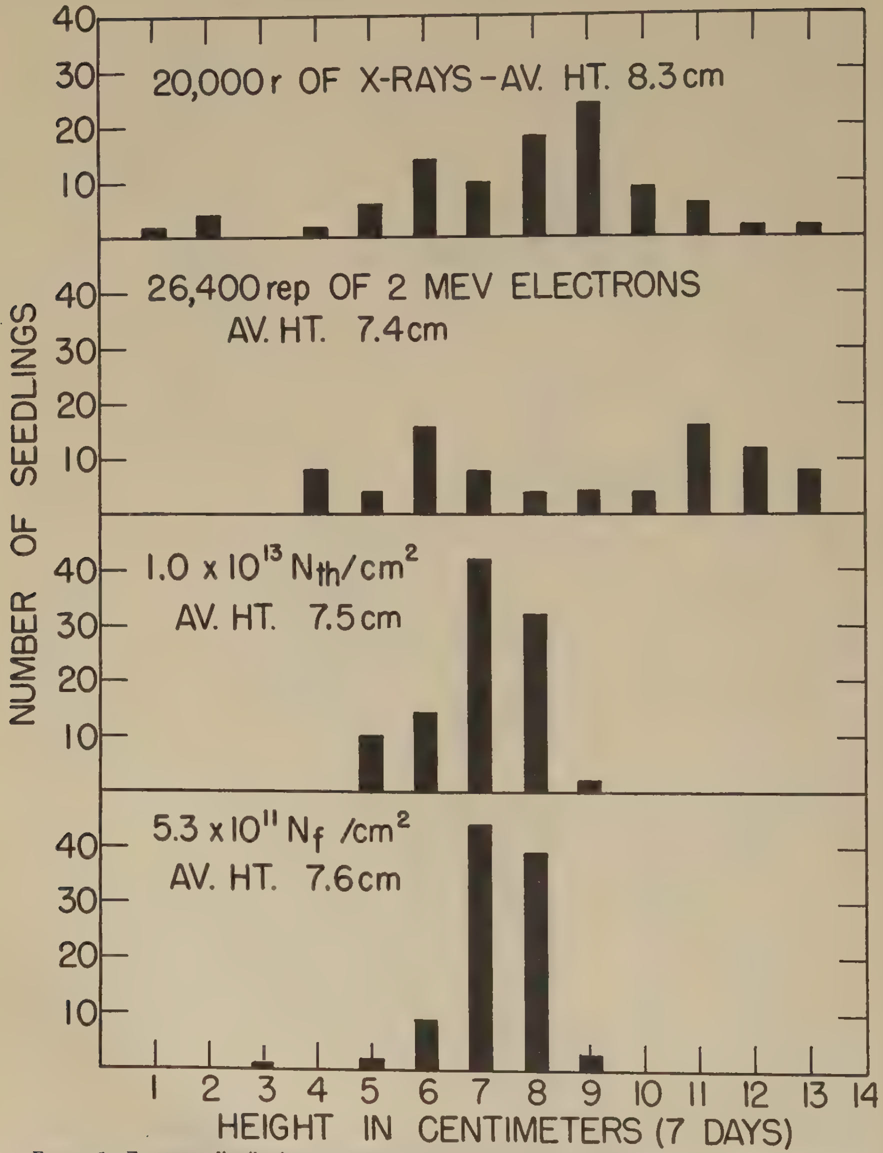


FIGURE 5. Frequency distribution of seedling heights from a dose of X rays, 2-mev electrons, thermal neutrons, and fast neutrons that reduce the average height to a comparable degree.

average inhibition of seedling growth were graphed (FIGURE 5). This figure lucidly illustrates the similarity between the curves obtained with thermal and fast neutrons. From these seedling studies, it seems logical to conclude that the differences observed are related to the physical characteristics of the radiations and, probably, of their specific ionization.

Effects of Ionizing Radiations on Survival

When attempts are made to grow to maturity plants that originate from irradiated seed, a large number of plants that would survive under more favorable conditions succumb, due to environmental adversities. This circumstance tends to mask differences between populations, but it can be mitigated if extreme care is taken to rid the soil in which the plants are grown of harmful microorganisms by sterilizing it with steam, or otherwise, especially if, in addition, temperature, illumination, and other growth conditions are maintained at an optimal level.

Under favorable conditions it was found that the uniform response of seeds to thermal neutrons, as compared with their response to X rays, as demonstrated with the seedling studies, was reflected in the number of plants that survived to maturity. This finding is illustrated in FIGURE 6, where it can be seen that there is a much sharper demarkation point between survival and death

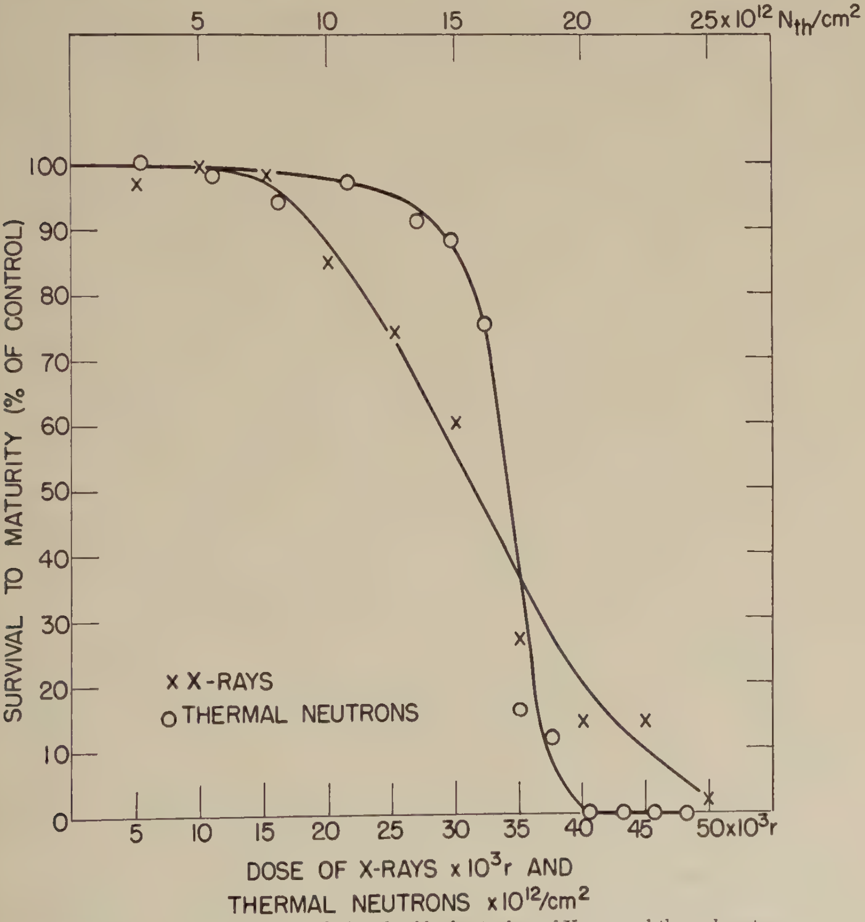


FIGURE 6. Relation of survival of seeds of barley to dose of X rays and thermal neutrons.

of seeds receiving graduated doses of thermal neutrons than there is in the seeds receiving graduated doses of X rays.

Effects of Ionizing Radiations on Chromosomal Interchange Frequencies

It has been demonstrated (Caldecott *et al.*, 1952) that, with thermal neutrons, it is possible to obtain higher frequencies of chromosomal aberrations in root tip cells of barley per unit inhibition of seedling growth, than it is with X rays. This fact suggested that X rays must be effecting their influence on chromosomal or on extrachromosomal constituents of the cell in a different way than were thermal neutrons. For this reason, studies were initiated to determine the nature of the chromosomal anomalies which persisted to meiosis in plants grown from irradiated seed.

The analyses were conducted at first metaphase on 1866 spikes from X-rayed progenies and on 1745 spikes from thermal neutron-treated progenies. With both sources of radiation, the only types of chromosomal anomalies that persisted to meiosis were those involving reciprocal interchanges, or other rearrangements, that left the cells with full chromosome complements, exclusive of minute deficiencies which, in this material, would be difficult or impossible to detect. Statistical analyses suggested that there might be a difference in the relative frequency in which two of the complex interchange types were produced, but, in the main, the types and relative frequencies of chromosomal alterations were the same for both kinds of radiation (TABLE 1). Within any one class of aberration, the frequency per cell of that aberration fitted a poisson distribution.

The fact that cells with a full chromosome complement persist to meiosis in far larger numbers than do chromosomally deficient cells indicates that the latter are "outcompeted," or are greatly retarded, or die during the ontogeny of the plant. This indication has added significance when it is realized that barley haploids may produce vigorously growing plants (Konzak, unpublished). It suggests that genic imbalance may be a factor contributing to the inability of deficient cells to compete with normal cells.

With both types of radiation, one interchange type occurred with what may be regarded as an unexpectedly high frequency, if it is assumed that reciprocal interchanges occur at random between arms of both homologous and nonhomologous chromosomes. This class of interchange has previously been called "pseudo-iso-chromosomes" by Caldecott and Smith (1952). This interchange, the fourth most common type observed, resulted from reciprocal exchange between major portions of the nonhomologous arms of homologous chromosomes (FIGURES 7, 15-18). Possible explanations for the high frequency of occurrence of this type of exchange may be that homologous chromosomes are positionally related to one another in cells of the dormant seed, or that there is a preferential rejoining between broken ends of homologs in comparison with nonhomologous chromosomes. Photomicrographs of some of the exchange types observed during the study are presented in FIGURES 8 to 18.

The frequencies of interchanges as a function of dose of both X rays and thermal neutrons are depicted graphically in FIGURE 19. Regression analyses indicated that the relationship was linear for X rays, and from considerations

Table 1. Frequencies of Interchange Types Persisting to Microsporogenesis from Dormant Seeds of Barley Treated with X-Rays and Thermal Neutrons.

Treatment	Spikes Analyzed No.	Type of Interchange												
		Ring-of-4		Ring-of-6		Two Rings-of-4		Two pseudo-iso-chromosomes[1]		Ring-of-4 + Ring-of-6		Ring-of-8		Others[2]
		Total No.	No. per 100 Spikes	Total No.	No. per 100 Spikes	Total No.	No. per 100 Spikes	Total No.	No. per 100 Spikes	Total No.	No. per 100 Spikes	Total No.	No. per 100 Spikes	
Control (No treatment)	173	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	
Dose of X-Rays														
5000 r	451	29	6.4	2	0.4	1	0.2	2	0.4	0	0.0	1	0.2	
10000 r	420	45	10.7	1	0.2	5	1.2	0	0.0	0	0.0	0	0.0	
15000 r	401	52	13.0	0	0.0	3	0.7	2	0.5	0	0.0	0	0.0	
20000 r	398	81	20.4	3	0.8	6	1.5	6	1.5	0	0.0	2	0.5	G
25000 r	196	45	23.0	5	0.3	3	1.5	2	1.0	0	0.0	0	0.0	
Total (X-Rays)	1866	252	13.5	11	0.6	18	1.0	12	0.6	0	0.0	3	0.2	1
Dose of Thermal Neutrons														
2.3×10^{12}	151	15	9.9	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	
3.0×10^{12}	150	25	16.7	2	0.1	0	0.0	0	0.0	0	0.0	0	0.0	
4.1×10^{12}	150	38	25.3	2	0.1	3	2.0	2	1.3	0	0.0	0	0.0	
5.5×10^{12}	149	35	23.5	2	0.1	0	0.0	1	0.7	0	0.0	0	0.0	
6.5×10^{12}	150	38	25.3	2	0.1	3	2.0	2	1.3	0	0.0	0	0.0	
7.5×10^{12}	150	37	24.7	5	0.3	5	3.3	4	2.7	0	0.0	0	0.0	
11.5×10^{12}	150	50	33.3	12	0.8	4	2.7	0	0.0	0	0.0	2	1.3	
12.2×10^{12}	150	57	38.0	4	0.3	2	1.3	4	2.7	2	1.3	1	0.6	C
14.2×10^{12}	150	51	34.0	5	0.3	10	6.7	1	0.7	2	1.3	1	0.6	B
15.5×10^{12}	128	47	36.7	11	8.6	13	10.2	4	3.1	2	1.6	1	0.8	2A
20.0×10^{12}	117	38	32.5	7	6.0	6	5.1	2	1.7	3	2.6	2	1.7	D,E,F
23.0×10^{12}	150	55	36.7	15	10.0	6	4.0	1	0.7	1	0.6	5	3.3	A,B
Total (Thermal Neutrons)	1745	486	27.9	67	3.8	52	3.0	21	1.2	10	0.6	12	0.7	9
Combined Total (X-Rays + Thermal Neutrons)	3784	738	19.5	78	2.1	70	1.8	33	0.9	10	0.3	15	0.4	10

[1] Interchanges resulting from exchanges between opposite arms of homologous chromosomes (see Caldecott & Smith, 1953).

[2] The letters designate the following types of interchange:
A - Ring-of-4 plus two pseudo-iso-chromosomes
B - Two rings-of-4 plus two pseudo-iso-chromosomes
C - Three rings-of-4
D - Ring-of-4, ring-of-6 plus two pseudo-iso-chromosomes
E - Two rings-of-6
F - Ring-of-4 plus ring-of-8
G - Ring-of-10.

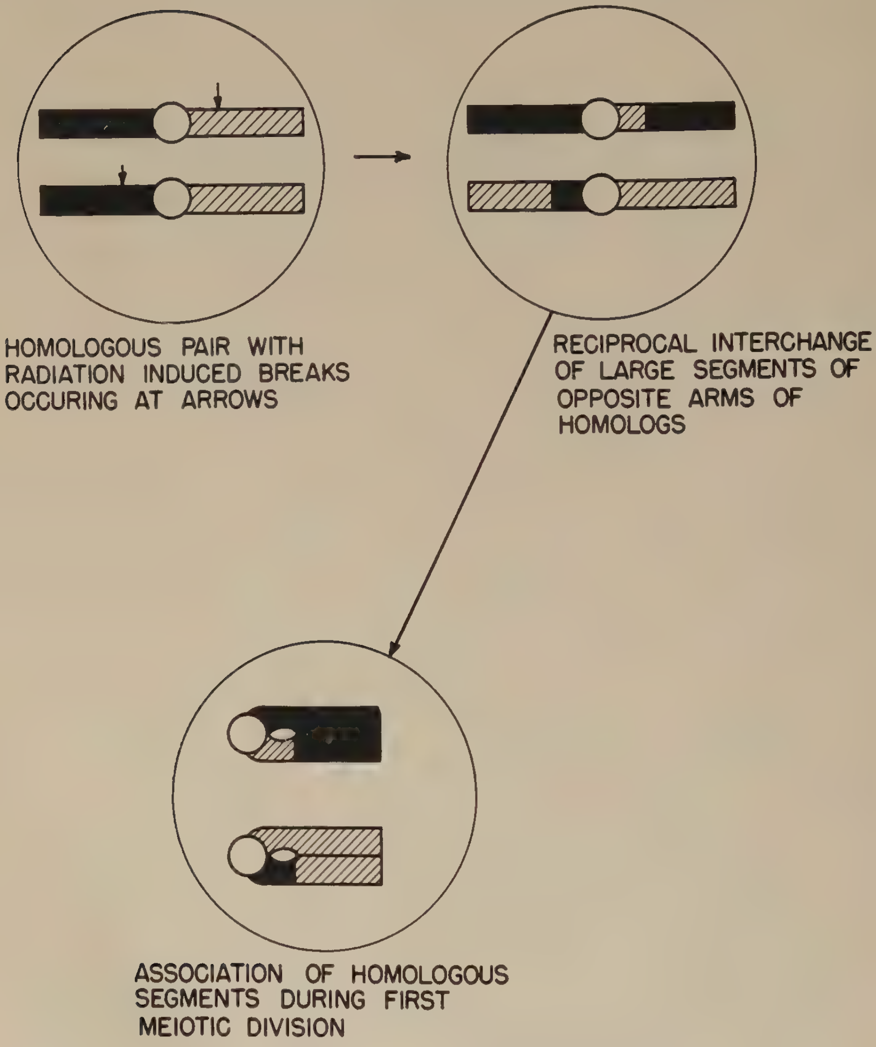

HOMOLOGOUS PAIR WITH RADIATION INDUCED BREAKS OCCURING AT ARROWS
RECIPROCAL INTERCHANGE OF LARGE SEGMENTS OF OPPOSITE ARMS OF HOMOLOGS
ASSOCIATION OF HOMOLOGOUS SEGMENTS DURING FIRST MEIOTIC DIVISION

FIGURE 7. Probable mechanism of pseudoisochromosome formation.

not presented here, was also linear for thermal neutrons (*cf*. Caldecott *et al*., 1954).

The thermal neutron data are in agreement with the findings of Giles (1940, 1943), who has pointed out that densely ionizing radiations apparently "simultaneously" break two chromosomes which have a good probability of exchange, thus resulting in the observed linearity. Similarly, the linear relationship obtained between interchange frequency and dose of X rays is in agreement with the findings of Sax and Brumfield (1943), who used both dry and germinating seeds of *Vicia* in their studies. However, these data are at variance with the exponential relationship between dose of X rays and frequency of interchanges observed by Sax (1941), using *Tradescantia* microspores, and Wolff (1954), using soaked *Vicia* seeds.

Wolff is of the opinion that the discrepancy between his data and those of Sax and Brumfield is due to the fact that a large proportion of the anaphase bridges scored by Sax and Brumfield as two-hit aberrations were in reality the result of one-hit isochromatids. The validity of this explanation is open to serious question because only a single anaphase bridge could result from a one-hit isochromatid break. When dormant seeds are irradiated, however, or when seeds which have been soaked but in which the chromosomes have not become "effectively" bipartite, single bridges are rarely observed; pairs of bridges are the rule (*cf.* Caldecott and Smith, 1952). Such pairs of bridges could arise only from a dicentric chromosome. It seems more likely that the apparent contradiction between the findings of Wolff (1954) and Sax (1941), on the one hand, and of Sax and Brumfield (1943) and Caldecott *et al.* (1954), on the other hand, is probably related to the organisms studied, particularly to the condition of the cells at the time of radiation. The fact that Sax (1941) used actively metabolizing microspores with a high water content and that Wolff did his work with soaked seeds suggests that at least some of the chromosome breaks are related to the presence of water. The implication is that chromosome fracturing was to some degree mediated by "indirect" effects.

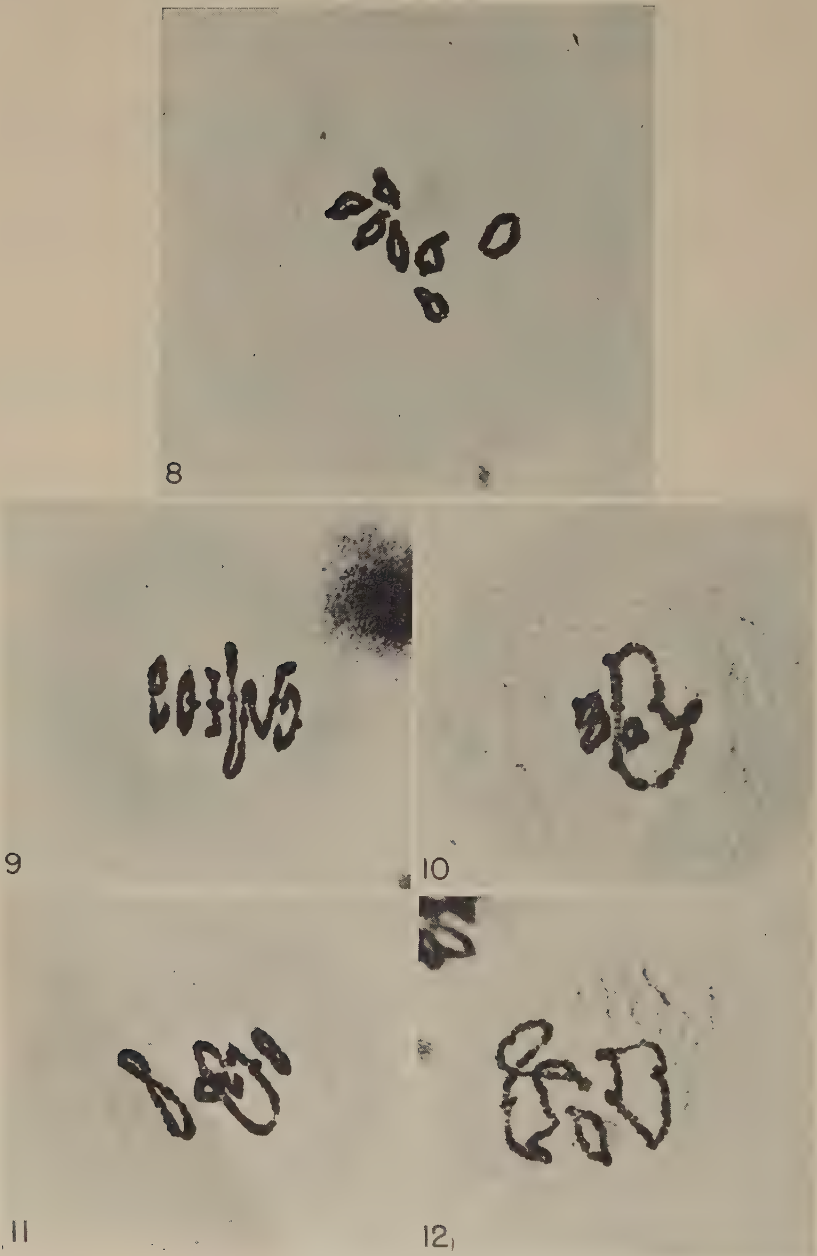
The time that the *Vicia* seeds were soaked is not specified by either Sax and Brumfield (1943) or by Wolff (1954), but in our work (unpublished), we have found that the sensitivity of soaked seeds to X rays is dependent on both the time which they are soaked and the temperature of the steeping water. Possibly the differences could be resolved if "standard" conditions of germination and treatment were used.

Further evidence that there is actually a linear relationship between dose of X rays and the frequency of interchanges observed in dormant seeds is derived from the fact that the same result has been obtained by examining seeds for dicentric interchanges at the first cycle of mitotic divisions and, much later in development, at first metaphase of meiosis, when they can be scored for monocentric reciprocal interchanges (*cf.* Caldecott *et al.*, 1954). This technique of double checking interchange frequencies, which involve both monocentrics and dicentrics, has proved to be invaluable in our studies.

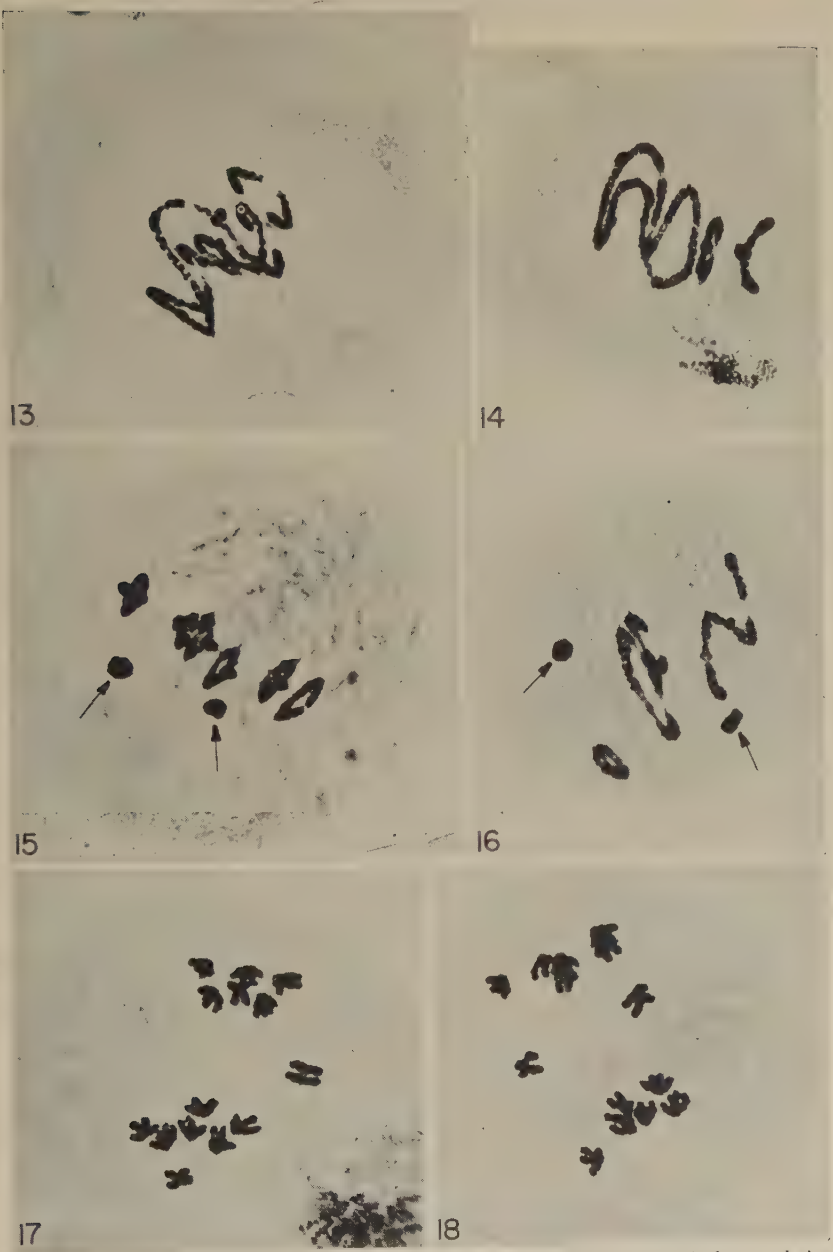
To obtain a linear relationship between interchange frequency and dose of X rays, as was found in this study, apparently requires that the breaks be produced in a manner similar to those produced by densely ionizing radiations. That is, that two breaks occur "simultaneously" in a geometrical locale conducive to exchange. If this occurrence does account for the observed linearity, it would lead to the conclusion that, in the dormant seed, the most effective part of the electron path, resulting from absorption of X-ray quanta, must be the densely ionizing tail.

Relation of Survival to Interchange Frequency

Counts were made on the survival to maturity of plants receiving different doses of X rays and thermal neutrons. The interchange frequency, plotted



Figures, 8 to 12. Chromosomes at microsporogenesis. Figure 8, seven bivalents (normal cell); Figure 9, four bivalents and chain-of-six; Figure 10, three bivalents and ring-of-eight; Figure 11, two bivalents, ring of four, and ring-of-six; Figure 12, one bivalent, ring-of-four, and ring-of-eight. (Figure 8 at diakinesis, others at first metaphase).



FIGURES 13 to 18. Chromosomes at microsporogenesis. FIGURES 13 and 14, two bivalents and ring-of-ten; FIGURE 15, six bivalents and two pseudo-isochromosomes; FIGURE 16, two bivalents, ring and chain-of-four and two pseudo-isochromosomes; FIGURE 17, one lagging pseudo-isochromosome at first anaphase; FIGURE 18, two lagging pseudo-isochromosomes at first anaphase (17 and 18 from the same spike as 15). FIGURES 13 to 16 are at first metaphase.

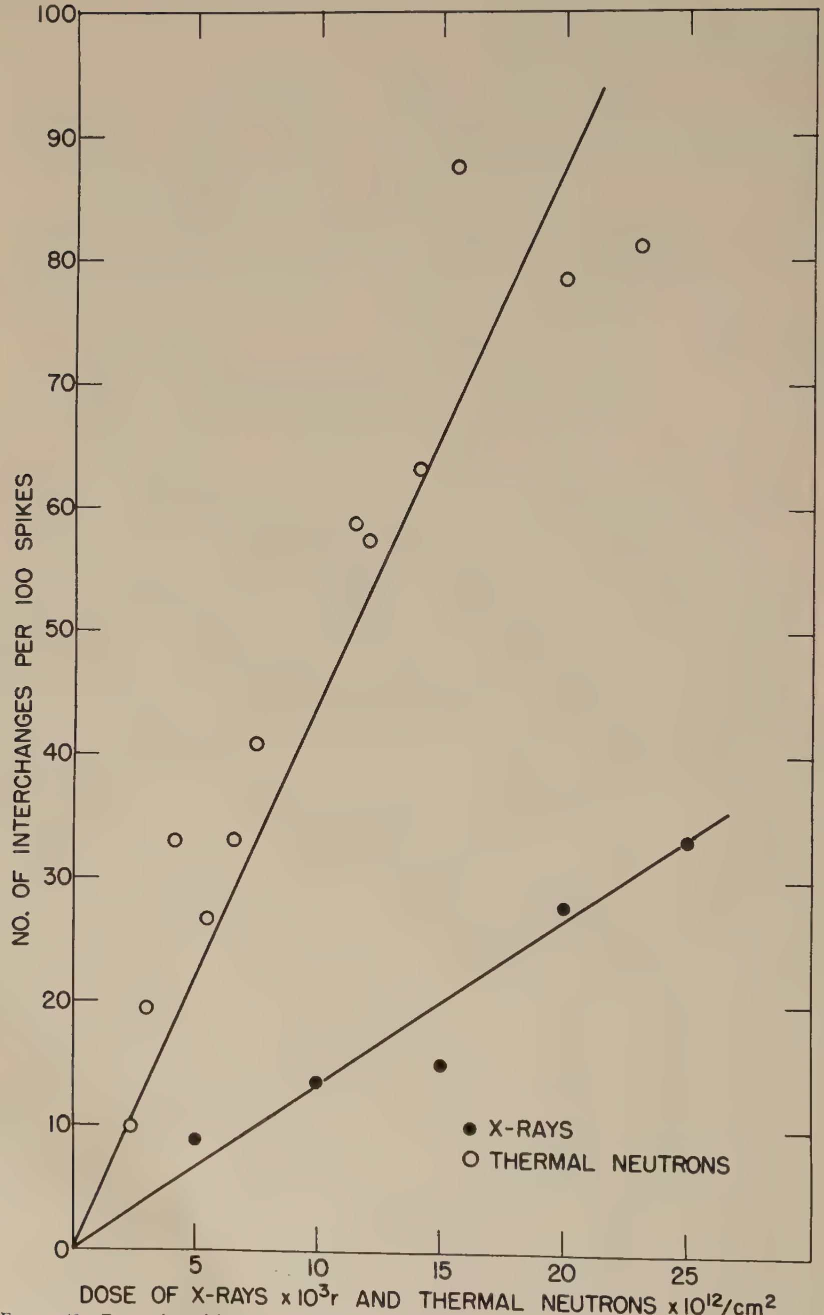


FIGURE 19. Regression of interchanges in microsporocytes on dose of X rays and thermal neutrons.

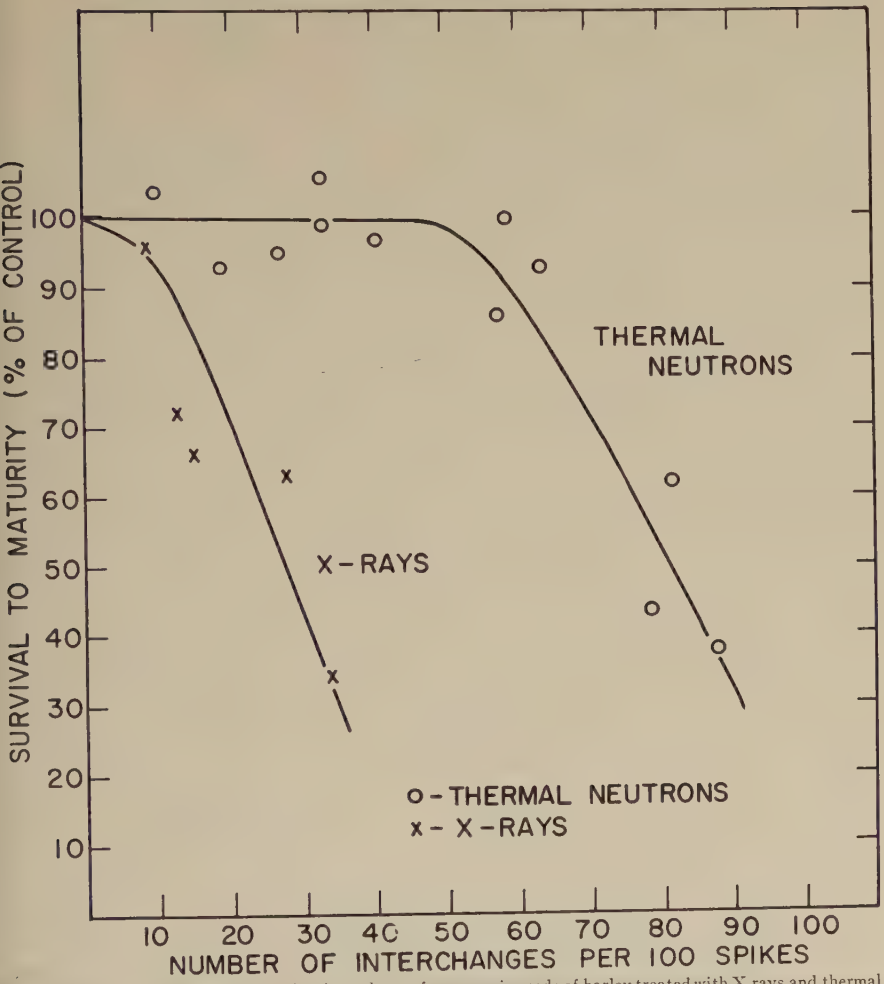


FIGURE 20. Relation of survival to interchange frequency in seeds of barley treated with X rays and thermal neutrons. (From Caldecott *et al.* 1954. Genetics. **39:** 240–259.)

as a function of survival, was then determined for each dose (FIGURE 20). This relationship clearly demonstrates that higher frequencies of interchanges were obtained, per unit survival, with thermal neutrons rather than with X rays. The same relationship has also been demonstrated for mutations (*cf.* Caldecott *et al.*, 1954). This finding raises the question as to the role played by genetic damage in the killing of the plants by X rays. It is apparently less than for thermal neutrons, insofar as can be detected with conventional genetic and cytogenetic analyses. It is impossible to state whether or not X rays may cause killing by affecting the genetic apparatus in an undetected manner. However, if one assumes that a genetic alteration would not go undetected, it seems logical to conclude that a greater proportion of the lethal action of X rays is caused by nongenetic events than is the case with thermal neutrons.

Effects of Ionizing Radiations on Mutation Frequencies

Plants grown from seeds surviving treatment with a range of doses of X rays and thermal neutrons and from the same populations on which meiotic interchange frequencies were determined were allowed to mature. From two to four spikes were removed from each plant, and the seeds were planted in the greenhouse for seedling mutation studies. The mutations observed resulted primarily from chlorophyll deficiencies, although other morphological variants were obtained. Statistical analyses indicated that there might be a difference in the relative frequencies in which two of the mutant types were produced by the two sources of radiation. However, most of the mutants were produced in the same relative frequencies by both sources of radiation (*cf.* Caldecott *et al.*, 1954). This finding indicates that there was no differential action on the mutation process by the radiations, and that all mutant types that can be obtained with X rays can also be produced with thermal neutrons.

For both sources of radiation, a linear relation between dose and frequency of mutations was obtained (FIGURE 21). This relation should not be interpreted, however, as meaning that the mutations do not result from structural chromosomal alterations, because it has also been shown that interchanges are linearly related to the dose of both X rays and thermal neutrons.

The Relation of Interchanges to Mutations

It is known that, in a number of species, detectable chromosomal anomalies such as deficiencies, duplications, or other alterations in the structural polarity of the chromosome may affect genic expression. For this reason, it was of interest to relate the frequency of mutations to the frequency of interchanges induced by different doses of X radiation and thermal neutron radiation (FIGURE 22). The result clearly demonstrates that, per interchange, about as many mutations were induced with X rays as with thermal neutrons.

If "point" mutations result from more subtle changes at a particular locus (*e.g.* slight chemical or sterochemical changes in the gene) than do chromosome breaks, because of their lower specific ionization, X rays should produce more point mutations relative to chromosome breaks than thermal neutrons. As it is apparent that they do not, the data suggest that both radiations induced these changes in the same way, and that the mutations observed resulted from the production of numerous ion pairs at a locus. This argument can be taken to support the findings of Stadler and Roman (1948) and of McClintock (1951, 1953), as well as work of others who have suggested that mutations result from intrachromosomal or interchromosomal rearrangement.

Summary

Data have been presented which show, in terms of growth inhibition and survival, that radiations with a high specific ionization (thermal neutrons and fast neutrons) more uniformly affect irradiated dormant seeds of barley than radiations with a low specific ionization (X rays and 2-mev electrons).

It was also shown that, when dormant seeds are irradiated, there is a linear relationship between the frequency of chromosomal interchanges and dose

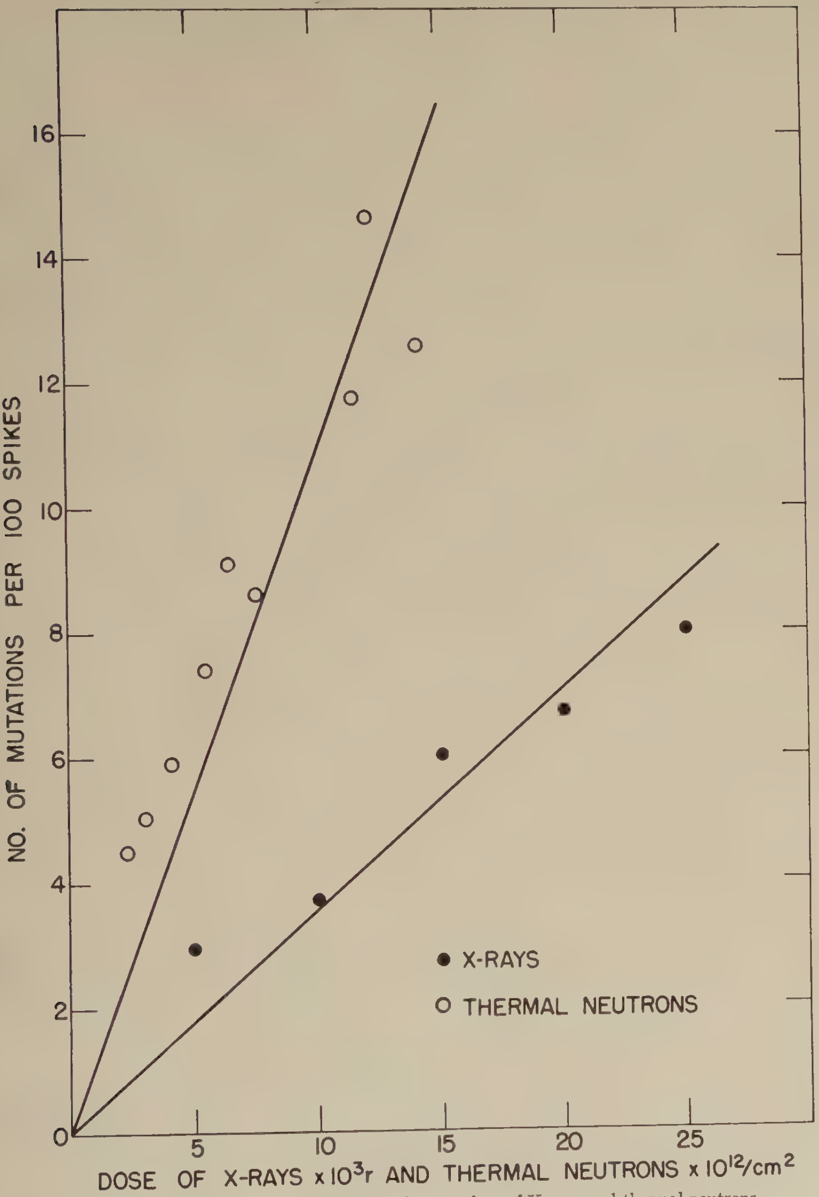


Figure 21. Regression of seedling mutations on dose of X rays and thermal neutrons.

of both X rays and thermal neutrons. It was suggested that, with X rays (as has been previously postulated for densely ionizing radiations), the linearity results from densely ionizing electron tails which produce "simultaneous" fracturing of two chromosomes which have a good probability of exchange.

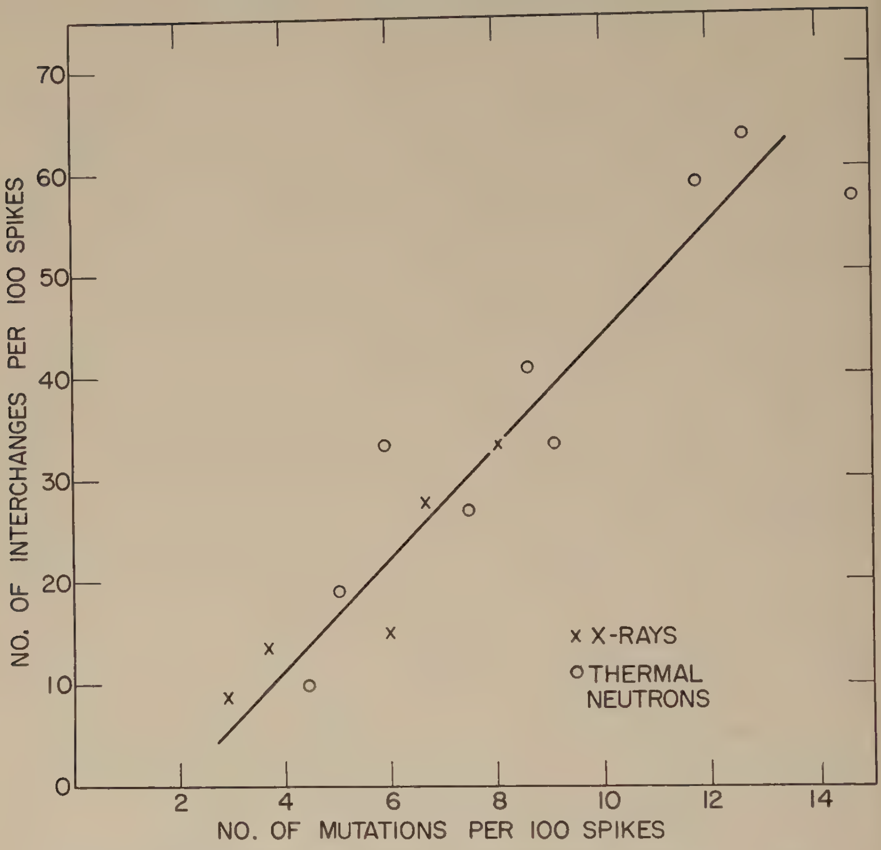


Figure 22. Regression of interchange frequencies on seedling mutation frequencies. (*Cf.* Caldecott *et al.* 1954. Genetics. **39:** 240–259.)

The inference is that, when dormant seeds are X-rayed, relatively few chromosomes are indirectly broken by active radicals mediated through water. With both kinds of radiation, the frequency of mutations as a function of dose was also linear.

Per unit survival, more than twice as many interchanges resulted from irradiating dormant seeds with thermal neutrons than with X rays. This finding was offered as evidence that X rays either affect the genetic apparatus in an undetected manner or produce events of an extrachromosomal nature which are largely responsible for death of the organism.

For both X rays and thermal neutrons, there was a linear relationship between the frequency of seedling mutations and the dose. In addition, per chromsome break (based on the frequency of interchanges), as many mutations were induced with X rays as with thermal neutrons. These observations were offered as evidence that the two kinds of radiation induced mutations in the same way, and that the mutations observed probably resulted from numerous ion pairs at a locus, possibly having their origin in intrachromosome or interchromosome rearrangements.

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COMPARATIVE STUDIES OF X RAY-INDUCED FORWARD AND REVERSE MUTATION*

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Soon after Muller (1927) first clearly demonstrated that ionizing radiations are capable of inducing mutations in *Drosophila melanogaster*, the question of the reversibility of such changes was raised. Evidence obtained in subsequent experiments with X rays by a number of investigators (Patterson and Muller, 1930; Timoféeff-Ressovsky, 1933) was taken to indicate that reverse mutations did indeed occur. In studies involving changes at single loci in *Drosophila*, the results appeared to be consistent with the hypothesis that X rays could induce reverse mutation of gene mutations of either spontaneous or X ray-induced origin.

Recently certain aspects of this concept of the action of ionizing radiations on genetic materials have been questioned. Experiments performed by Lefevre (1950) repeating certain of the earlier studies in *Drosophila* have yielded negative results in that no reversions were obtained following X-ray exposures. In addition, Stadler has carried out comprehensive studies on mutation at the A_1 locus in maize which have a direct bearing on the problem of the nature of X ray-induced genetic changes. Cytogenetic analyses of X ray-induced A losses (Stadler and Roman, 1948) indicated that the majority of such losses were not transmissible through the male gametophyte. In the three cases having normal or nearly normal transmission, analyses demonstrated that small deletions were present in each instance. Hence no evidence was obtained of other than destructive effects of X rays on the genetic material at this locus. Furthermore, experiments were performed in an attempt to induce by X rays reverse mutational changes from the recessive a (colorless) to the dominant A (purple) phenotype (Stadler, 1944). In these tests, the a mutant used was one known to undergo reversion to A under the influence of the Dt gene. In extensive tests, no X ray-induced reversions were obtained.

The conclusions which have been drawn from these experiments with *Drosophila* and maize are that ionizing radiations, such as X rays, produce, in addition to gross chromosomal rearrangements, essentially only destructive changes such as deletions, and are incapable of inducing positive, dominant gene mutations. If, indeed, essentially all X ray-induced effects simulating gene mutations are destructive and result in gene loss, this means that such changes would be genetically stable and incapable of subsequent change either spontaneously or with additional mutagenic treatments. As indicated by Lefevre, the accumulation of a great many such irreversible mutations in a population would have serious evolutionary consequences. Hence, more information on the characteristics of X ray-induced gene mutations is of potential practical as well as theoretical importance.

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The present discussion will be concerned with studies of X ray-induced mutations in the mold *Neurospora crassa*. Most of the investigations to date have been concerned with the problem of reverse mutation at specific genetic loci. In addition, certain preliminary comparative studies dealing with X ray-induced forward and reverse mutation at one locus will be considered. These studies are concerned with two distinct but clearly related problems. The primary problem is to determine whether X rays can induce dominant mutations of positive action. The experiments bearing on this point are similar in principle to those of Stadler and Lefevre in that attempts have been made to produce reverse mutations at specific loci, similar to the change from recessive *a* to dominant *A* in maize. In the event such changes are in fact produced, the additional problem of the precise genetic nature of such changes must then be considered.

In order to facilitate the presentation of the experimental data, the results obtained at the two different loci which have been the subject of most of these investigations will be discussed separately. These two types of mutants are mutants requiring inositol, referred to as inositolless, and mutants requiring adenine which also produce a purple pigment, referred to as purple-adenineless.

The advantages as well as details of the experimental techniques in the use of biochemical mutants of *Neurospora* in studies of spontaneous and induced reversions have been presented in detail elsewhere (Giles, 1951; Jensen, Kirk, Kølmark, and Westergaard, 1951) and will be indicated here only briefly. Biochemical mutants having specific growth factor requirements have major advantages in the study of reversions, since such changes to growth factor independence are effectively self-selecting from among a large population of unchanged cells under appropriate screening conditions. In *Neurospora*, in addition to ease of detection, the resulting mutant types can be subjected to a relatively rigorous genetic analysis. Under appropriately controlled experimental conditions, the reversion technique can be utilized to investigate, both qualitatively and quantitatively, mutational changes at individual loci. Additionally, the use of strains carrying mutations at two or more loci permits precise comparative studies of the relative effectiveness on individual genes of the same mutagen under identical treatment conditions.

Inositolless Mutants

Previous studies (Giles, 1951) of a series of inositol-requiring mutants (produced either by ultraviolet or by nitrogen mustard) indicated that such mutants could be induced to revert upon subsequent exposure to ultraviolet. Genetic evidence supported the view that the majority of such reversions could be most simply interpreted as reverse gene mutations. Similar reversion studies with some of these mutants were carried out using X rays, and positive evidence for X ray-induced reversions was also obtained. More detailed physiological, biochemical, and genetic investigations of these mutants have now been performed, and certain of these results will be reported here.

Evidence for X ray-induced reversions. For the two mutants investigated in most detail (numbers 37401 and 89601), there is clear evidence that X ray-induced reversions do occur. The spontaneous rate of reversion in both mutants is very low, although such reversions occur occasionally. Following X irradiation, the rate of reversion is markedly increased. The relation between X-ray dose and reversion rate in microconidia is shown in FIGURE 1 and appears to be approximately linear on the basis of the available data, especially for mutant 37401.

Phenotypic characteristics of reversions. In general, as indicated in a previous discussion of ultraviolet induced reversions (Giles, 1951), two major categories of reversions can be distinguished phenotypically, especially in experiments in which tests are performed with the colonial, microconidiating, fluffy strains. The first category includes reversions which are, in general, phenotypically

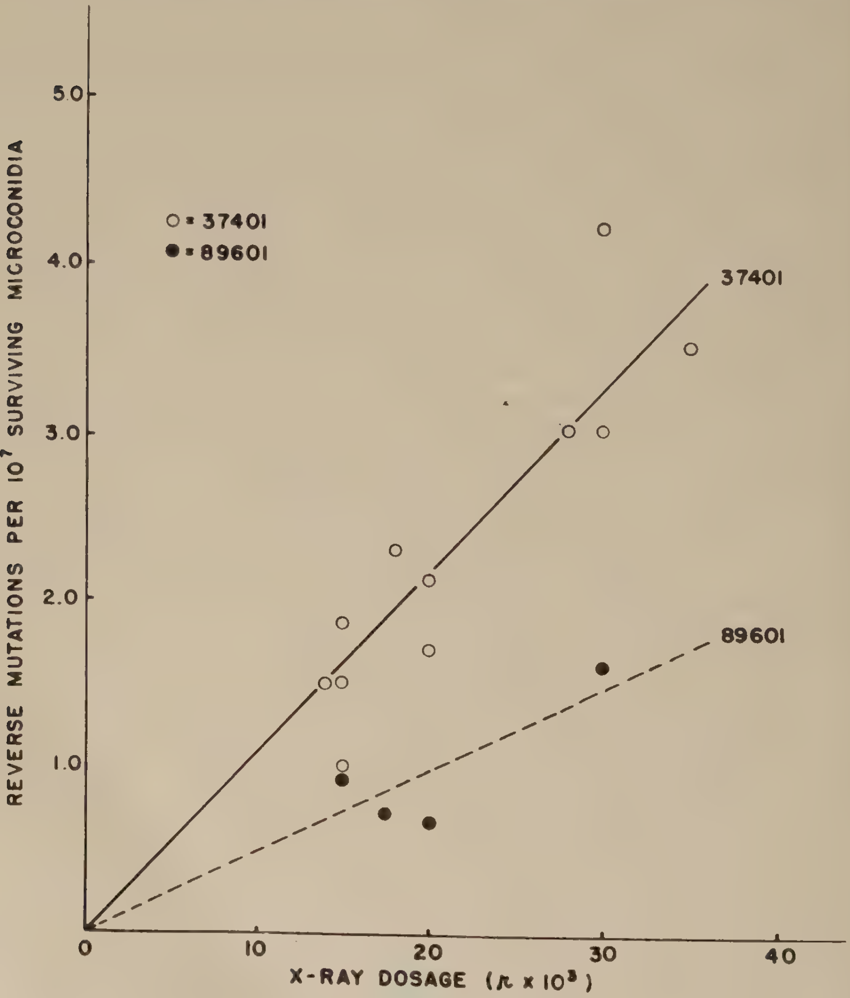

FIGURE 1. Relation between X-ray dosage and frequency of reverse-mutations in two inositolless mutants (in *cmf.* stocks).

similar to the parental strain in morphology and growth rate. These reversions usually appear within about six days after irradiation. The second category includes reversions which are markedly different phenotypically from the parental strain, having a much restricted growth rate and a more colonial morphology. These types typically appear considerably later than the first type and with a much lower frequency. The distinction between these two categories is admittedly somewhat arbitrary and, in the case of X ray-induced reversions, it becomes less distinct than for reversions produced with ultraviolet. With X rays, there appears to be more phenotypic variation in the first category, since a larger proportion of somewhat intermediate, relatively slow-growing types occurs.

Genetic evidence, which will be discussed later, indicates that, in general, the first category of reversions consists of complete or essentially complete reverse mutations, while two types of mutants are present in the second category: partial reverse mutants (which behave as alleles at the inositolless locus) and suppressor mutants (which are nonallelic to inositolless mutants). An explanation for the rather marked phenotypic variation of revertants nominally included in category 1 was not clear, however, since, if such reversions resulted from single gene changes involving precise reverse mutation, it would be anticipated that they would be phenotypically uniform and similar both to the original parental type and to closely related inositol-independent cultures. Considerable attention has accordingly been given in this study to reversions with markedly slower growth rates, chosen from extreme types apparently belonging in category 1 as well as from mutants belonging clearly in category 2. Comparative tests of such types indicated immediately that they could be separated into two cateogries on the basis of their response to inositol supplementation of the minimal medium: those which are not, and those which are markedly stimulated by inositol. The great majority of reversions are not stimulated (at least to any marked degree) by inositol, regardless of whether they have normal or slow growth rates on minimal. These correspond in general to reversions in category 1. Stimulation by inositol of revertants having normal growth rates essentially the same as inositol-independent types would not be anticipated on the hypothesis that these represent exact reverse mutation to the wild type condition. Some explanation, however, must be sought for the slow-growing reversions, which are not stimulated. On the assumption that these represent reverse mutations, as the genetic evidence indicated, at least two alternative explanations seemed possible. Either they were reverse mutational types in which reverse mutation at the inositolless locus resulted in an allele characterized by a restricted growth rate, even with inositol supplementation, or the restricted growth rate was the result of an additional simultaneous mutation at another locus affecting growth rate and independent of the reverse mutation to inositol independence.

These alternative possibilities may be distinguished by crossing such slow-growing reversions with an inositolless culture as closely related as possible to the culture in which the reversions were produced. The results of such crosses are given in TABLE 1. In these comparisons, all tests have been made of growth rates on minimal supplemented with 7 μg. of inositol/ml. All cultures

TABLE 1

SEGREGATION FOR GROWTH RATE AND INOSITOL REQUIREMENT IN COMPLETE ASCI FROM CROSSES BETWEEN X RAY-INDUCED REVERSIONS (INOSITOL-INDEPENDENT STRAINS) IN MUTANT 89601 (CULTURE 89601-CL15-7A) AND A SISTER INOSITOLLESS STRAIN (CULTURE 89601-CL15-25a)*

Cross	Linear growth rate (mm./da. on 7 μg. inositol/ml.) and inositol requirements†						
$P_1 \times P_2$	Parental types		Segregation types				
	P_1	P_2	Ascus No.	Ascospore numbers			
				1 or 2	3 or 4	5 or 6	7 or 8
25a × 7A	9.1−	10.0−	1	10.4−	11.0−	11.0−	11.2−
			11	9.8−	10.0−	10.0−	10.2−
25a × 7A-R1-88	9.7−	10.9+	9b	10.0−	10.7+	11.0+	9.8−
	11.0−	12.2+	20	12.2+	9.5−	9.0−	11.6+
25a × 7A-R11-87	11.0−	4.4+	2	5.1+	6.6+	10.0−	10.0−
	9.7−	4.2+	10	10.2+	9.8−	3.8+	3.9−

* All cultures of cmf. morphology (Giles, 1951); growth rate of i^+ (W.T.) in this morphology ($cmfi^+$) averages ca. 10.3 mm./da.

† + = Inositol independence; − = inositol dependent.

are of the colonial, microconidial, fluffy genotype. The first cross involves the two parental inositolless strains. These are sister cultures, and they have similar growth rates on minimal essentially equivalent to that of normal inositol-independent cultures in this morphology. Resulting growth rates, indicated for segregants from two complete asci from this cross, are representative of several additional asci tested. The second cross involves a reversion with a normal growth rate. Here the resulting segregants have similar growth rates regardless of their inositol requirement. The final cross is the one of particular interest, in that it involves a slow-growing reversion. In this instance, it is evident that the slow growth habit does not always remain associated with inositol independence, but that recombination can occur, yielding an inositol independent type with a normal growth rate. Evidently, in this reversion, an independent mutation affecting growth rate had occurred. Tests of additional reversions having different growth rates gave generally similar results although, in some instances, the pattern indicated that more than one major factor influencing growth rate was segregating.

In addition to clear-cut differences in growth rate, reversions also often differed from the parental strain in certain morphological features. An objective evaluation of the segregation pattern for such differences is difficult, however, and it is not yet clear how often such differences arise from mutational events unassociated with reverse mutation at the inositolless locus. It is possible, in fact, that all reversions may differ in some constant respect, aside from their inositol independence, from the original parental strain, and accordingly that, in this sense, exact reverse mutation does not occur. More precise methods of comparison will be required before a decision on this point can be reached with the present material. It is clear from the growth rate results just discussed, however, that much of the phenotypic variability noted in these reversions is ascribable to independent mutational events, either spontaneous or induced, which are unrelated to the specific reversion to growth-factor independence.

The growth characteristics of reversions which are stimulated by inositol may be indicated by a comparison of two such types, both induced in inositolless 89601. Comparative tests in growth tubes of one of these, which subsequent genetic tests indicated to be a partial reverse mutation, are reproduced in FIGURE 2. This partial reverse mutation grows on minimal, but at a rate less than one tenth that of wild type or of a complete reverse mutation (in this comparison, mutant 89601-CL15-7A-R1-88 was used in wild type morphology). With appropriate inositol supplementation, however, it attains the wild type growth rate.

Similar comparisons, but in these tests using dry weight as a measure of growth, have been made for another reversion, which subsequent genetic tests indicated to be the result of a suppressor mutation. These results are reproduced in FIGURE 3. In this instance, the reversion grows appreciably on minimal, having a dry weight about one fifth that of wild type at the six-day interval. Supplementation with inositol stimulates growth markedly and, at a level of 4.0 μg. inositol/ml., the growth curve is quite similar to that of wild type. The methods used in making these tests are described by Partridge and Giles (1953).

It is clear from these comparisons that all inositolless reversions are not identical phenotypically or physiologically. The details of further evidence for genetic differences among these types will be discussed subsequently.

Evidence for inositol synthesis by X ray-induced reversions. The simplest hypothesis to explain the inositol-independence of reversions is that such mutants have regained the ability to carry out the synthesis of this growth factor. It is possible, however, even though highly unlikely, that reversions may be types which no longer require inositol either of exogenous or endogenous origin. Since one of the questions involved in this discussion concerns the possibility of X ray-induced changes of positive effect, it seemed very desirable to determine whether such reversions are indeed synthesizing inositol. Quantitative estimations of inositol activity in mycelial pads and filtrates were made by agar plate assays, using inositolless *Neurospora crassa* (strain 37401) and *Ashbya gossypii* as test organisms, as described by Partridge and Giles (1953). The presence of inositol was confirmed by measurement of its mobility on paper chromatographic strips, as revealed by application of the strips to large assay plates seeded with inositolless *Neurospora*. The results of plate assays of mycelia and filtrates of wild type, mutant 89601, a complete reversion, a partial reversion, and a suppressed mutant (in this instance from an ultraviolet experiment with mutant 37401) are given in TABLE 2. Of these strains, only the inositolless mutant received inositol supplement (1.75 μg./ml.). The presence of inositol was demonstrated in all strains. It was found that the mycelial contents were closely similar for the complete revertant and wild type, while the mutant mycelium contained about half that concentration. The same level was reached by the partial revertant only after 10 day's growth;

that of the suppressed mutant remained low even after 21 days. Only the wild type and the complete revertant filtrates contained inositol. The complete revertant, however, apparently excreted only about one fifth as much inositol as the wild type.

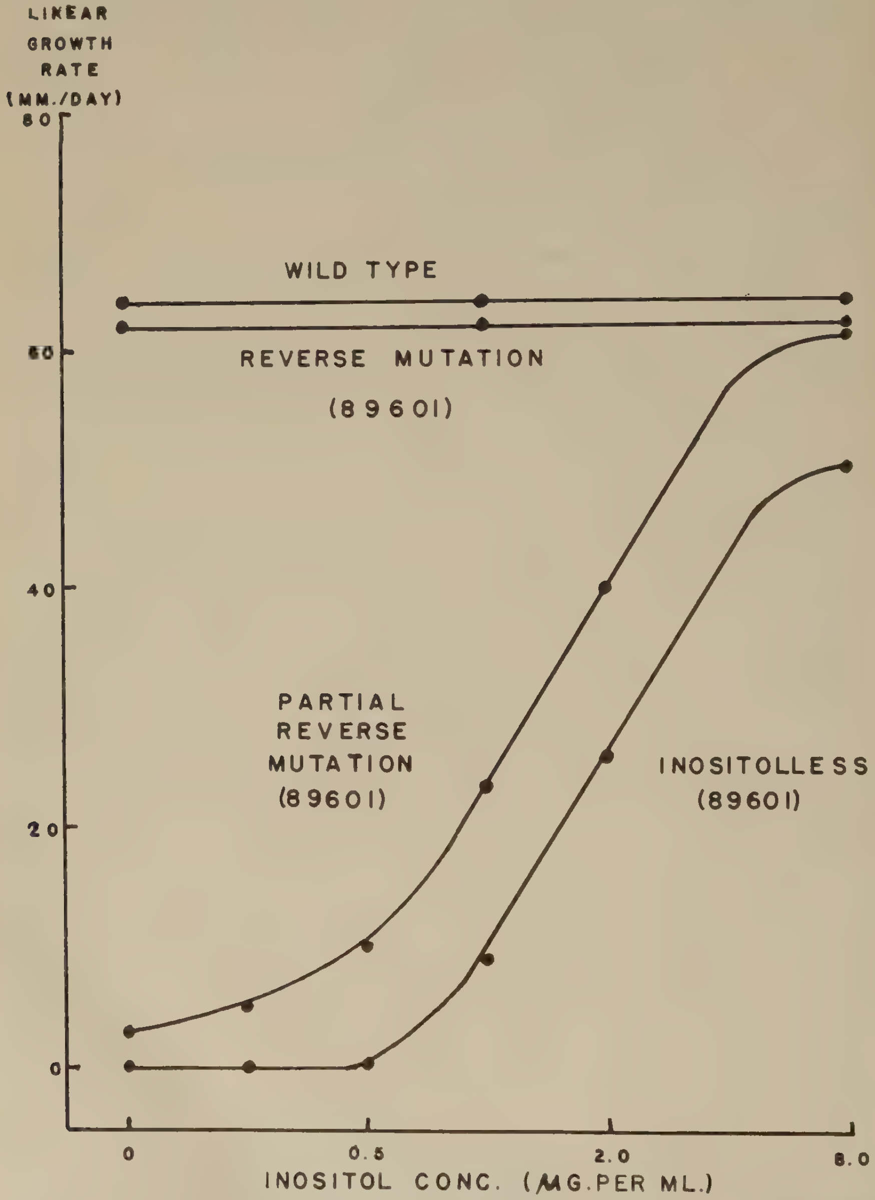

FIGURE 2. Linear growth rates with increasing inositol supplements of a complete and a partial reverse mutant (induced by X rays in inositolless mutant 89601) compared with wild type and inositolless 89601. All strains with wild type morphology.

Dominance of X ray-induced reversions. The previous evidence has shown clearly that X ray-induced reversions give rise to a positive biochemical effect in that they result in a restoration of inositol synthesis. Such an effect would presumably be dominant to the original mutant condition of inositol dependence. Here again, however, it is desirable to test such an assumption. In *Neurospora*, dominance cannot be tested in the heterozygous condition, since no diploids have yet been produced in this mold, as has been done in *Aspergillus* (*cf.*, Pontecorvo, 1953). However, heterocaryons can be utilized for this purpose. Since most of the reversion experiments already discussed were performed with uninucleate microconidial stocks, which usually yield homocaryotic reversions, such mutants do not give information on this point. In certain earlier experiments with ultraviolet, however, reversions were produced

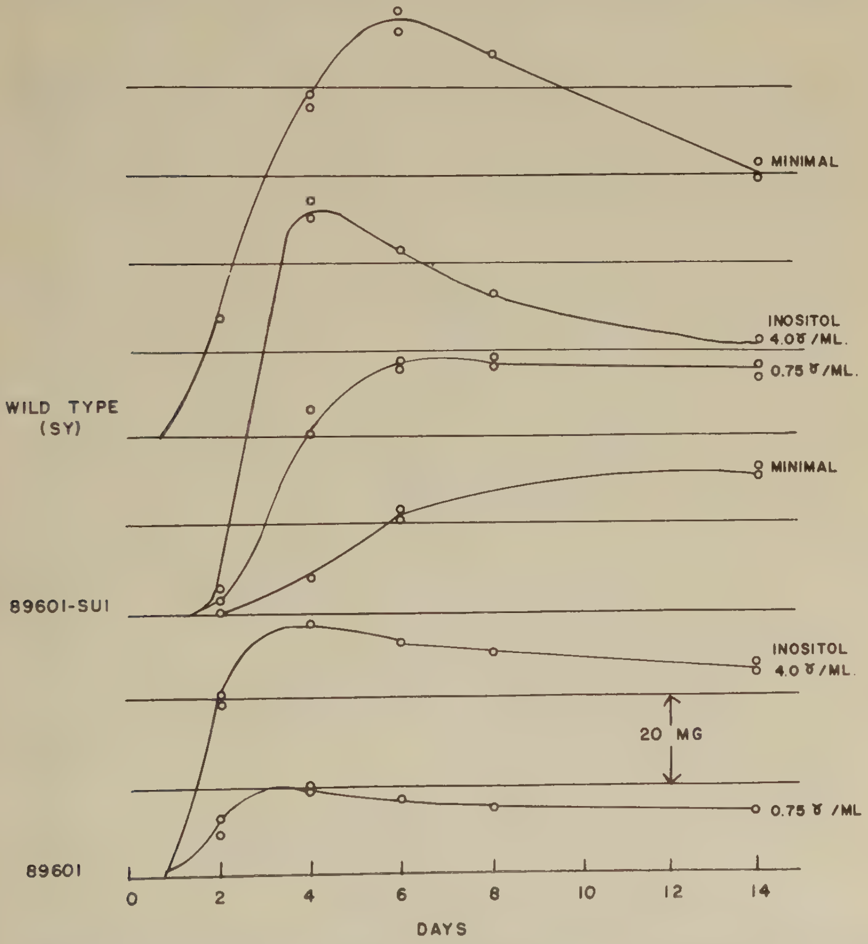

FIGURE 3. Variations of mycelial dry weights of inositolless strains over a 2-week period on 20 ml. liquid medium in 125 ml. Erlenmeyer flasks, supplemented with inositol as noted in figure. Numbers at left identify strains used in curves beginning on same base lines. Each curve represents duplicate observations. All strains with wild type morphology.

TABLE 2

INOSITOL ACTIVITY IN MYCELIUM AND FILTRATES OF NEUROSPORA AS DETERMINED BY TWO TEST SPECIES IN SIMULTANEOUS AGAR PLATE ASSAYS*

Strain tested			Growth conditions		Inositol activity			
					Mycelium µg./mg. dry wt.		Filtrate mg./flask	
Type	Strain No.	Morph.	Growth period (days)	Inos. Suppl. (mg./flask)	Neur.	Ashbya	Neur.	Ashbya
Wild type	SY	w.t.†	5	0	2.0	2.8	0.045	—
	SY	w.t.	12	0	2.0	2.8	—	—
Mutant	89601	w.t.	5	0.44	1.1	—	0.005	0.015
Complete reversion (X ray)	89601 (R1-88)	w.t.	5	0	2.0	—	0.01	—
Partial reversion (X ray)	89601 (R4-85)	w.t.	5	0	0.7	—	0.0	—
		w.t.	11	0	1.1	—	0.0	—
		cmf‡	11	0	0.8	0.8	0.001	0.018
Suppressed mutant (U.V.)	37401 Su-1	w.t.	21	0	0.2	0.2	inhib.	inhib.

* Figures are based on triplicate determinations at each of two levels. Replicates of standard and unknown pads were placed on separate assay plates. Assay organisms were *Neurospora crassa* inositolless mutant 37401 and *Ashbya gossipyi* A.T.C.C. No. 8717. Inositol-dependent strains were grown on the sub-maximal level of inositol selected for chromatographic studies (1.75 µg./ml.). Growth at 25° C.

†w.t. = wild type.

‡ cmf = colonial, microconidial, fluffy stock (Giles, 1951).

in macroconidial strains. Tests indicated clearly that such types were heterocaryons and grew on minimal, accordingly showing in these cases that inositol independence was dominant to inositolless. In order to be certain that X ray-induced reversions behaved in a similar fashion, experiments with macroconidial strains (utilizing mutant 37401) were also performed. Presumptive heterocaryotic reversions were induced by exposing macroconidia to X rays. From each of three such reversions, growing on minimal, two hyphal tip isolations were made, the resulting cultures permitted to conidiate, and conidial platings carried out in minimal medium supplemented with inositol and containing sorbose to restrict growth. In each instance, a sample of the resulting colonies was isolated and tested, with the results shown in TABLE 3. The recovery of both inositol-independent and inositolless cultures established, in each instance, that heterocaryotic reversions had been produced and that X ray-induced inositol independence is dominant to inositol dependence. It may also be noted that the nuclear ratios, as measured by macroconidial platings, appear to be rather constant within a given reversion, as judged by tests of separate hyphal tips, whereas this ratio is quite different in reversion 8 as compared with reversions 4 and 5.

Genetic nature of induced reversions. The preceding evidence establishes quite clearly that X rays can induce dominant mutational changes of positive effect in *Neurospora*. The detailed genetic nature of such changes remains to be determined, however. As has been indicated in previous discussions of ultraviolet induced reversions (Giles, 1951), two principal types of change might be anticipated—either reverse mutations or suppressor mutations. In addition, the possibility of more complex changes involving rearrangements

TABLE 3

TESTS FOR THE RECOVERY OF INOSITOLLESS CULTURES FROM REVERSIONS INDUCED BY X RAYS IN MACROCONIDIA OF AN INOSITOLLESS MUTANT (STRAIN 37401-CL21-2a)

Reversion No.	Hyphal tip No.	Test of colonies isolated from macroconidial platings	
		Inositol-indep.	Inositolless
R4-Y107	A	8	31
	B	12	28
R5-Y107	A	16	4
	B	33	7
R8-Y107	A	38	2
	B	20	0

giving rise to position effects must be considered. If suppressors are involved, crosses of reversions to wild type should yield inositolless recombinants unless the suppressor is very closely linked with the inositolless locus. Tests of a large sample of X ray-induced reversions have been made (Giles, 1951) to detect suppressor mutations. In all tests of reversions classified in category 1, that is, as phenotypically rather similar to the parental type, no inositolless recombinants were obtained. Tests of reversions in category 2, which are slow-growing types of quite different morphology, have proved more difficult to make, since these reversions often fail to cross easily. Certain of these reversions, however, have proved to be suppressors, as, for example, is the case with one of the two reversions described earlier which are stimulated by inositol. In this instance, ascus segration types similar to those described previously for an ultraviolet-induced suppressor (Giles and Partridge, 1953) were obtained.

Initial observations and preliminary genetic tests of both ultraviolet and X ray-induced reversions led to the expectation that all reversions markedly stimulated by inositol would prove to be suppressors, whereas those not so stimulated would represent reverse mutations (or, at least, not suppressors). This has not proved to be uniformly true, however. In one instance an X ray-induced, slow-growing reversion stimulated by inositol, when crossed with wild type, has failed to yield inositolless recombinants in tests of more than 300 random spore isolates. This reversion, whose growth-characteristics were described earlier, is hence considered to be a partial reverse mutation to an intermediate allele at the inositolless locus resulting in a partial restoration of inositol synthesis, since inositol concentration remains growth-rate-limiting. Clearly, such a type could also be regarded as a very closely linked suppressor, but the distinction between these two possibilities by this type of genetic analysis eventually becomes impracticable if linkage is sufficiently close.

The major category of reversions, those phenotypically rather similar to the parental type and not markedly stimulated by inositol, remains to be considered. On the simplest hypothesis, these reversions represent complete reverse gene mutations to inositol independence which may or may not be accompanied by independent mutations affecting growth rate or other characters.

TABLE 4

LINKAGE DATA FOR INOSITOLLESS AND X RAY-INDUCED REVERSE MUTANTS OF INOSITOLLESS 89601

Cross No.	Strains crossed	No. asci tested	cent. *iv i p asp** % recombination			
			cent-*iv*	*iv-i*	*i-p*	*p-asp*
1	W. T. – 1 × iv i p asp	27	11.1	25.8	3.7	13.0
2	W. T. – 2 × iv i p asp	26	17.3	30.8	3.8	25.0
3	i al_2 × iv p asp al_2^+	30	15.0	28.4	1.7	18.3
4	Reverse mutation – 1 × iv i p asp	41	20.7	39.0	6.1	28.0
5	Reverse mutation – 2 × iv i p asp	28	25.0	33.8	1.8	25.0
6	Reverse mutation – 3 × iv i p asp	27	21.0	20.0	7.4	24.1
7	Partial reverse mutant × iv i p asp	59	15.2	30.4	5.8	26.3

* Symbols: *iv* = isoleucine-valineless (16117); *i* = inositolless (37401); *p* = paraaminobenzoicless (1633) *asp* = asparagineless (S-1007); W. T. = wild type.

It is also possible, however, that such reversions might result from rearrangements giving rise to position effects. If such rearrangements are of large size, they should result in disturbed linkage relationships and be detectable on this basis. Linkage tests have already been made of certain ultraviolet-induced reversions, and these tests have failed to disclose evidence for gross rearrangements. Similar tests have now been conducted with selected X ray-induced reversions (in mutant 89601), and the results are indicated in TABLE 4. In general, the linkage data for the three complete reversions and the partial reversion are similar to those obtained from the control crosses involving wild type strains. Thus, these data suggest that gross rearrangements are not present in the particular instances studied. It should be noted, however, that the reversions were produced in mutant 89601 and crossed for linkage tests with mutant 37401. This procedure was adopted since stocks with appropriate linkage markers were available in mutant 37401, and extensive crossing tests of the two mutants had given evidence that they were allelic or, if pseudoallelic, that they gave very few inositol-independent recombinants. Recently, additional linkage tests with a new histidineless marker proximal to the inositolless locus and much closer than the isoleucine-valineless mutant (as judged by linkage data from mutant 37401) have indicated that there may be differences in the linkage relationships of these two inositolless mutants with this marker. In view of these results, any interpretation of the previous linkage data, although compatible with the view that gross rearrangements are not involved in these reversions, must be considered provisional. If mutant 89601 does prove to differ in its linkage relationships, this difference may indicate that it is a particular type of inositolless mutant, possibly having arisen in conjunction with a rearrangement. It is of interest to recall that comparative ultraviolet-X ray studies indicated that at comparable microconidial survival levels of about 20 to 30 per cent, mutant 89601 reverts much more readily with X rays than with ultraviolet, whereas the reverse is true for mutant 37401 (Giles, 1951). The possibility that some type of rearrangement mechanism is involved in some or all of these reversions produced by X rays in mutant 89601 cannot yet be excluded.

Linkage studies with X ray-induced reversions in mutant 37401, utilizing the newly available more closely linked proximal marker have recently been initiated. The results obtained to date with random ascospore isolations indicate no significant linkage differences in comparisons of a control cross of this mutant with crosses of three X ray-induced reversions.

Purple-Adenineless Mutants

Somewhat similar, although not as extensive studies have been carried out with X ray-induced reversions in purple-adenineless mutants of *Neurospora.* This type of mutant, which was first extensively used in reversions experiments by Kølmark and Westergaard (1949), has the advantage of being phenotypically distinguishable without testing, since the block in adenine synthesis arising from mutation also results in the accumulation of a distinctive purple pigment. Reversions arising spontaneously or induced by ultraviolet or chemicals have resulted uniformly, in experiments performed to date, in the simultaneous restoration of adenine independence and loss of pigment formation.

Experiments with X rays utilizing purple-adenineless mutant 38701 (originally produced by ultraviolet) indicate clearly that ionizing radiation induces reversions to adenine independent, nonpurple types phenotypically similar to wild type and to reversions produced by other mutagens. Comparative tests to determine the relative frequencies of X ray-induced reversions to inositol indpendence and to adenine independence have been performed with a double mutant requiring both inositol and adenine (mutant numbers 37401 and 38701). Using a colonial, microconidial, fluffy stock, reversions to adenine independence were found to occur approximately 3.5 times as frequently as those to inositol independence at the single dose level tested, 30,000 r.

Chemical tests were performed to determine whether such revertants were synthesizing adenine, as would be expected. These tests involved the following procedure: the freeze-dried and powdered mycelium was hydrolyzed for one hour at 100° C. in 1 N HCl to liberate free purines (Markham and Smith, 1950). Samples of the solutions eqivalent to *ca.* 750 μg. dry weight were chromatographed on Whatman No. 54 paper with 1 per cent aqueous ammonia. For comparison 5 μg. adenine sulfate hydrate and 5 μg. guanine hydrochloride were run simultaneously. Mixtures of the adenine with the unknowns were also run. Rf values of 0.32 for adenine and 0.40 for guanine were observed by ultraviolet absorption and by silver chromate staining (Reguera and Asimov, 1950). Both adenine and guanine were detected in the mycelium of mutant, revertants, and wild type in comparable amounts. These results indicate that, with adenineless, as with inositolless reversions, X ray-induced mutations have a positive effect, in that essentially normal adenine synthesis has been restored in the revertants.

Tests were also performed to determine whether X ray-induced adenine independence is dominant to adenine dependence. For this purpose, heterocaryons were utilized, the procedure being essentially the same as that outlined previously for inositolless reversions. The results of tests of three adenine-

TABLE 5

TESTS FOR THE RECOVERY OF PURPLE-ADENINELESS CULTURES FROM REVERSIONS INDUCED BY X RAYS IN MACROCONIDIA OF PURPLE-ADENINELESS MUTANT 38701 (CULTURE NO. 8743-CL20-3a)

Reversion No.	Hyphal tip No.	Tests of colonies isolated from macroconidial platings	
		Adenine-independent	Purple-adenineless
R1-Y107	A	32	1
	B	33	0
	C	33	0
R2-Y107	A	10	23
	B	7	27
	C	10	23
R3-Y107	A	33	1
	B	31	2
	C	32	2

independent strains induced by X rays in macroconidia are shown in TABLE 5. In all three instances, both adenine-independent and adenineless colonies were recovered, demonstrating that adenine independence is dominant to adenine dependence.

Extensive genetic tests of these X ray-induced reversions of adenineless 38701 have not yet been performed. Preliminary tests indicate, however, that suppressor mutations are not involved. These revertants are thus considered to represent true reverse mutations, but additional linkage tests will be necessary to provide further evidence on this point.

The Reversibility of X Ray-Induced Mutants.

In all the experiments discussed so far, the mutants studied were produced either by ultraviolet or by chemicals. The fact that X rays can produce reversions of such mutants can be taken as evidence that all genetic effects of X ray are not destructive. Alternative explanations of these X-ray effects are possible, however, depending on the genetic nature of the original mutations. More definite evidence concerning the nature of X-ray effects should be obtainable from studies of the potential reversibility by X rays of mutants originally induced by X irradiation. Earlier investigations in *Neurospora* utilized already available biochemical mutants for reversion studies since, until recently, the isolation of forward mutations, especially of a specific type, was a much more laborious procedure than producing reversions. However, the recent development of effective concentration techniques (Woodward *et al.*, 1954) has made the isolation of forward mutations, at least of certain types, more feasible. Hence, experiments were recently initiated (de Serres, unpublished) to produce appropriate mutants by X irradiation, in order to study their reversibility. The results of these preliminary experiments will be summarized briefly.

Attempts were made to produce with X rays both inositolless and purple-

TABLE 6

EXPERIMENTS WITH X RAY-INDUCED PURPLE-ADENINELESS MUTANTS*

Mutant number	Genetic analyses of mutants crossed with wild type			Tests for X ray-induced reversions to adenine-independence†				
	% ascospore germination	% adenineless segregants	% recombination with mating type locus‡	Total viable conidia tested in both control and X ray series ($\times 10^6$)	No. of reversions		Reversions/10^6 viable or surviving conidia	
					Control	X ray	Control	X ray
M1	90.0	43.4	20.5	160.3	0	0	0.0	0.0
M4	71.0	3.5	20.0	123.0	0	0	0.0	0.0
M5	62.5	11.2	14.5	154.9	0	0	0.0	0.0
M8	34.5	32.0	13.5	160.0	0	1	0.0	0.008
M9	25.0	0.0	—	194.9	0	0	0.0	0.0
M2	91.0	48.2	9.4	127.2	20	30	0.16	0.30
M6	73.0	50.7	5.4	209.4	0	0	0.0	0.0
M10	89.0	55.1	8.2	117.0	1	9	0.009	0.07
M13	92.0	50.0	8.5	138.2	0	14	0.0	0.13

* de Serres, unpublished.

† X ray experiments: macroconidial stocks used; 25,000 r.; ca. 80% survival.

‡ Normal per cent crossing over with mating type locus ca. 8.1% (see text).

adenineless mutants in a wild type macroconidial strain. This strain, 74A-4b, and also strain 73a-10a, which was used in the preliminary genetic analysis, were obtained from Doctor Patricia St. Lawrence of Yale University, and were derived by morphological and cytological selection of progeny from crosses of Emerson's strains E-5256A and E-5297a (Emerson and Cushing, 1946). To date, no inositolless mutants have been obtained, but nine purple-adenineless mutants of X-ray origin have been produced. In comparable control experiments, no spontaneous mutants of either type were detected; hence it appears safe to conclude that the purple-adenineless mutants are indeed of X ray origin. Initial genetic and reversion studies performed with these nine mutants are summarized in TABLE 6. The genetic analysis has consisted of crossing the mutants with wild type 73a-10a and testing ascospores isolated at random with respect to mating type and adenine requirement (as detected by purple pigmentation). It is immediately apparent that the mutants comprise two categories. In the first group, either segregation of adenineless or recombination with mating type, or usually both, are abnormal. In the second group, both these processes appear to be normal or approximately so. The recombination value between the purple-adenine and the mating type loci used as a standard in these comparisons is *ca.* 8.1 per cent based on data for the purple-adenineless mutant 35203 (Barratt *et al.*, 1954; Houlahan *et al.*, 1949) derived by ultraviolet treatment of strain 1A and 25a (Beadle and Tatum, 1945). When experiments were carried out to determine whether X rays could induce reversions in these mutants, a striking parallelism was found between their genetic behavior and their reversibility to nonpurple, adenine independent types. Mutants in group 1 appear to be essentially stable, with the exception of one revertant in M 8 which may represent a contaminant. In group 2, however, reversions were obtained in three of the four mutants. These reversions were of both spontaneous and induced origin. Genetic analyses have not yet been made of these reversions. It appears probable, however, that they are not the result of suppressor mutation, since no suppressors of purple-adenineless mutants have yet been obtained in extensive tests of reversions arising spontaneously or induced by ultraviolet or by chemicals.

A final evaluation of these results must await additional evidence. In particular, it will be necessary to obtain more evidence concerning the linkage relationships of various purple-adenineless mutants, utilizing additional genetic markers. It does appear probable, however, that X ray-induced purple-adenineless mutants result from more than one type of genetic change. The abnormal genetic behavior of mutants in group 1 makes it seem likely that these types either resulted from, or are associated with some type of chromosomal rearrangement. Their relative stability suggests either that structural changes giving purple-adenineless mutants seldom undergo reversion, or that such changes frequently result in simultaneous deletion of the adenine locus.

The apparently normal genetic behavior of mutants in group 2 makes it possible to consider these mutants as arising from changes restricted to the purple-adenineless locus or its immediate vicinity. The fact that three of these types can undergo reversion either spontaneously or following X-ray treatment makes it difficult to see how such "adenine losses" can represent simple deletions, as in the case of the "A_1 losses" studied by Stadler and Roman (1948). The one instance (M 6) of a stable mutant in this group may well be the result of a deletion. It is suggestive that, in this instance, there is a somewhat lower recombination with mating type, but the significance of this difference is not established.

Discussion

These results with *Neurospora* appear to stand in contrast with those reported in *Drosophila* and in maize. This contrast, however, may turn out to be more apparent than real. The numbers of individual flies tested by Lefevre in his *Drosophila* experiments dealing with reversions at specific loci were not very large, even though a few positive results would have been anticipated on the basis of earlier studies. With *Neurospora* mutants, however, the testing of much larger numbers is easily feasible, and induced reversion frequencies of the order of one per 10^7 surviving conidia, or even less, can be detected with relative ease. It is certainly to be anticipated that the testing of additional large numbers in *Drosophila* should yield some reversions, even if these turn out, upon testing, to be the result of genetic changes other than true reverse mutation. In addition, in the *Drosophila* studies, as pointed out by Lefevre, there is no evidence presented demonstrating that any of the genes at the various loci tested are capable of reversion. Critical evidence for the failure of X rays to induce reversion at a given locus, however, can be obtained only where it is possible to obtain reversions either spontaneously or by the use of other mutagens to insure that the mutant under consideration is not genetically stable.

With respect to the results obtained in maize, the a_1 locus may prove to represent a special situation, not necessarily as suitable for tests of X ray reversibility as it was first thought, since it has been suggested (McClintock, 1953) that reversions of *a* to *A*, which occur only when the Dt gene is present, may be controlled by a type of activator-dissociator system. Furthermore, the finding that deficiencies always accompany X ray-induced "*A* losses" which possess approximately normal male gametophyte transmission, does not necessarily mean that deletions are involved in all *A* losses. The elimination of a majority of such *A* loss mutants as a result of reduced pollen transmission could have stemmed, as Stadler and Roman (1948) point out, from mutational events such as gross chromosomal rearrangements independent of the *A* losses involved, which, in at least some of these instances, might be the result of true gene mutation. It appears possible that, in *Neurospora*, mutants of restricted viability comparable to the *A* loss mutants with reduced gametophyte transmission in maize might be subjected to even more stringent selection in this completely haploid organism. Thus the surviving forward, as well as reverse, mutants in *Neurospora* may represent a highly-selected sample of possible mutants.

The present results with *Neurospora* are not to be interpreted as indicating that X ray-induced genetic changes are comparable, in respect to the proportions of possible types of changes involved, with genetic changes induced by other mutagens or occurring spontaneously. It is clear from studies in *Drosophila* that X ray-induced sex-linked recessive lethal mutations are associated with gross chromosomal rearrangements in a much higher percentage of cases than is true of lethals induced by ultraviolet or chemicals, or of spontaneous origin (Slizynski *et al.*, 1947). Although comparable data are not available in *Neurospora*, it appears likely that a similar situation would exist in this organism for forward mutation and possibly for reversions as well. The evidence in *Neurospora* does appear to indicate that all localized genetic changes induced by X rays are not necessarily losses and hence irreversible.

Summary

Experiments on the production by X rays of reversions in inositolless and purple-adenineless mutants of *Neurospora*, induced originally by ultraviolet or by chemicals, indicate that X rays can induce dominant, positive mutations resulting in the restoration of specific biochemical syntheses. Genetic analyses indicate that at least two major mechanisms are involved in the origin or reversions—suppressor mutation and reverse mutation, and that reverse mutation is much more frequent. Although most reverse mutations in inositolless mutants appear to arise from complete or nearly complete reversions to the wild type condition and are not stimulated by inositol, one instance of a partial reverse mutation has been obtained in which the synthesis of inositol is still growth-limiting. Evidence has been obtained that at least some and possibly all slow-growing reversions not stimulated by inositol have arisen as a result of reverse mutation accompanied by independent mutations affecting growth rate. The results of linkage analyses of reversions included in the reverse mutation category are compatible with the view that many such changes are not associated with gross chromosomal rearrangements, but additional evidence is being sought on this point.

Preliminary studies of the reversibility by X rays of X ray-induced purple-adenineless mutants have been made. The results with nine purple-adenineless mutants of X-ray origin indicate that these mutants comprise two generally corresponding categories on the basis of their genetic bechavior and their X-ray reversibility. The five mutants in the first category show various indications of abnormal genetic behavior and are not induced to revert with X rays. The four mutants in the second category show essentially normal genetic behavior, and three of these undergo reversion either spontaneously or following X irradiation. The over-all results of these studies are interpreted to indicate that in *Neurospora* not all X ray-induced forward mutational changes are destructive, and that X rays are also capable of inducing positive, dominant changes, some of which are most simply interpreted as true reverse mutations.

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CYTOCHEMICAL STUDIES OF CHANGES INDUCED IN CELLULAR MATERIALS BY IONIZING RADIATIONS[*]

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The experiments summarized in this report stemmed from our efforts during recent years to determine the nature of the structural and functional derangements induced in living cells by ionizing radiations. Many studies directed to these ends have been concerned either with the initial impact of ionizing particles on cellular materials or with the terminal products of the reaction initiated thereby. Biologists have amassed considerable information about the types and frequencies of induced structural aberrations, but relatively little about the antecedent alterations in patterns of organization of cellular materials. The need for information of this type is emphasized by the accumulating evidence that many chemicals can simulate radiations in the production of aberrations. In some cases, the cytologically detectable responses of cells to X rays and to chemicals are strikingly similar. Thus, in studies of chromosomal rearrangements obtained by treatment of spermatozoa of *Drosophila*, it was found that breaks induced by either X rays or nitrogen mustard were distributed at random along the chromosomes, irrespective of their division into euchromatic and heterochromatic regions,[†] and that the frequency of rearrangements was increased to a similar extent (about 50 per cent) in each case, if the males were exposed to near infrared radiation before exposure to X rays or nitrogen mustard.[18]

Such experiments indicate that both physical and chemical agents can modify the same chromosomal materials, despite possible differences in the pathways by which the cytologically detectable structural modifications are realized. In an effort to identify these materials and determine the changes effected in their patterns of organization, cytochemical studies were initiated some years ago in which the action of crystalline enzymes on cellular materials was detected by suitable staining methods. The validity of these procedures for localizing nucleic acids and proteins and for determining their relations was established in critical experiments on sections and squashes of nonirradiated tissues.[7, 13, 17] By digesting the cells in nucleases or proteases, used independently or in succession, or in combination with chemical treatments, and staining subsequently in basic or acidic dyes, it was found that the chromosomes contain both ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) intimately associated with each other and with histones and nonhistone proteins.[19] The evidence presented below indicates that alterations effected in this complex nucleoprotein fabric by ionizing radiations can be detected by suitable cytochemical methods.

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[†] These data have been cited by some authors as evidence of preferential breakage in heterochromatic regions, but this interpretation rests on consideration of the frequencies of rearrangements detected by salivary-gland-chromosome analysis. There is abundant evidence, as summarized in Kaufmann,[12] that the breaks induced by irradiation of spermatozoa are distributed at random with respect to length of the euchromatic and heterochromatic regions in the chromosomes at the time of irradiation.

Observations

Methyl green stainability. Cells that have been irradiated often show adherent or clumped chromosomes. It has frequently been assumed, without adequate experimental evidence, that such changes result from depolymerization or nonpolymerization of DNA. Depolymerization of DNA in tissue sections by the enzyme desoxyribonuclease results in complete loss of stainability of the chromosomes with methyl green or the leucobasic fuchsin of the Feulgen technique. Methyl green stainability can be inhibited, however, without appreciable reduction of Feulgen stainability (for example, by short treatment of tissue sections with hot water), and this response has been attributed to depolymerization of DNA without its loss from the cells.[24, 28, 40]

Proceeding on the assumption that depolymerization of DNA could be detected by methyl green stainability, spectrophotometric determinations were made a few years ago, using the Pollister-Moses type of equipment,[29] of meristematic nuclei of onion roots that had been exposed to doses of X rays within the range from 200 r. to 16,000 r. No reduction in methyl green stainability, as compared with the controls, was effected by these treatments.[22] The results are in essential agreement with those of Sparrow, Moses, and Dubow,[32] who found no appreciable effect of 20,000 r. of X rays on methyl green or Feulgen stainability of pachytene nuclei of Trillium.[37] Such experiments led to the conclusion that X-ray doses sufficient to reduce the viscosity of solutions of DNA do not depolymerize this nucleic acid in the chromosomes of plant cells, but the reliability of methyl green stainability for determining the degree of polymerization of DNA in tissue sections has more recently been questioned.[1, 36, 38] * Although the objections raised by these critics may invalidate the premises on which the quantitative studies of methyl green stainability were formulated, the results obtained in the experiments cited above nevertheless indicate that heavy doses of X rays did not alter the DNA in a manner that could be detected by its capacity to bind this dye.

It would be premature to conclude from these spectrophotometric studies that ionizing radiations cannot induce changes in amount of DNA. Chemical analyses of extracts from liver cells of irradiated mice (600 r. of hard X rays), made in these laboratories by Paigen and B. N. Kaufmann,[27] showed parallel fluctuations in the levels of DNA and RNA at different time intervals after treatment. The spectrophotometric studies on plant cells, however, gave no indication of similar changes, although the treatments were sufficient to evoke adhesion and clumping of the chromosomes. The question then arose as to whether the modifications of substrate materials leading to such structural alterations can be detected by other cytochemical methods. In an effort to answer this question, the responses of irradiated cells to enzymatic hydrolysis were determined.

Effect of nucleases on irradiated and nonirradiated cells. The facility with which solutions of crystalline enzymes degrade specific substrate materials has been found to depend on their state of aggregation or pattern of association with other cellular components. As an illustration, hydrolysis of tissue sections for a short period of time in low concentrations of desoxyribonuclease removes all Feulgen-stainable material from interphase and early prophase nuclei, but not from the condensed chromosomes, although spectrophotometric and autoradiographic studies indicate that no significant increase in amount of DNA occurs during the prophases of somatic mitoses.* Proceeding on the assumption that changes in aggregation or pattern of association might be induced by ionizing radiations, experiments were undertaken to determine the action of desoxyribonuclease and ribonuclease on cells of irradiated and nonirradiated onion roots and grasshopper embryos. These experiments led to the conclusion that "irradiation may cause some dissociation of desoxyribonucleoproteins in the living cell,"[22] but the data on which that conclusion was based have not been published heretofore, and will be summarized at this time.

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* See also *Discussion* in Sparrow *et al.*[32]

Onion bulbs that had developed a heavy growth of roots were bisected longitudinally, and the halves placed in lucite-covered moist chambers. One half of each bulb, with the attached roots, was given a dose of X rays of either 300, 600, 1200, 2400, 4800, or 9600 r. The other half served as a nonirradiated control. Roots were removed from each of the halves at the end of the X-ray treatment and, at intervals thereafter, during a 24-hour period. The roots were fixed in acetic acid-alcohol, subjected to enzymatic hydrolysis, and stained in either methyl green, methyl green-pyronin, the leucobasic fuchsin of the Feulgen technique, or fast green.

Observations on the meristematic cells revealed that short periods of hydrolysis in desoxyribonuclease (0.001 mg. per ml. of 0.003 M $MgSO_4$, at pH 6) effected greater reduction of stainability with methyl green and the Feulgen reagent in the X-rayed roots than in the controls. This response was detectable about five hours after exposure to 300 r. or 600 r., but much sooner after the higher doses. There was no detectable difference between irradiated and nonirradiated cells in the effect of a low concentration of ribonuclease (0.001 mg. per ml. of water, at pH 6) on pyronin stainability of chromatin, unless pyknotic degeneration had occurred; but this concentration of the enzyme did eliminate pyronin colorability of cytoplasm more rapidly in the controls than in the irradiated roots. This latter response may be attributable, in part at least, to the fact that sections of irradiated roots which had not been subjected to enzymatic hydrolysis revealed a marked increase in pyronin stainability of the cytoplasm.

Stainability of meristematic cells with the acidic dye-fast green was, in general, also increased as a result of exposure to X rays. Treatment with desoxyribonuclease effected a further increase in fast green stainability of chromosomal materials, as compared with the 0.003 M $MgSO_4$ controls, and treatment with ribonuclease an increase in stainability of both nuclear and cytoplasmic materials, as compared with the water controls; but the amount of the increase appeared to be about the same in the irradiated and nonirradiated roots. Such enzymatically induced increases had previously been shown to be attributable to the degradation of nucleoproteins.[17]

In the studies on grasshopper embryos (*Melanoplus femur-rubrum*, *Tri-*

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* For summary of evidence, see Swift.[38]

merotropis maritima, and *Chortophaga viridifasciata*), comparisons were made between irradiated and nonirradiated (shielded) halves of the embryos, as well as between individuals removed from the same egg pod. Attention was focused in these studies on the response to enzymatic hydrolysis of irradiated cells showing "sticky" or clumped chromosomes. In such cells, methyl green and Feulgen stainability was reduced less rapidly by desoxyribonuclease, and pyronin stainability of the cytoplasm more rapidly by ribonuclease, than in the controls. Refractoriness to enzymatic hydrolysis of chromatin "agglutinated" by treatment of roots with chemicals has been reported by Yakar.[41]*

A major criticism of studies involving visual estimation of staining intensities, such as have just been described, is the subjective nature of the evidence they afford. In efforts to compensate for this handicap, the comparison ocular was used, and replicated series of coded slides were evaluated by two or more individuals. Such analyses were usually in very close agreement, and furnished reasonable evidence of the reliability of the method. Conceding the validity of the analytical procedure, it remains to be determined whether the observed changes are quantitative or reflect differences in patterns of association of constituent materials. The distinction is not always obvious from visual inspection, but the various lines of evidence outlined above, including the spectrophotometric studies of methyl green and Feulgen stainability, leave little question that many of the differences between the responses of irradiated and nonirradiated cells to enzymatic hydrolysis are due to alterations in patterns of organization of cellular materials.

Effect of tryptic digestion on irradiated and nonirradiated cells. Further evidence that ionizing radiations effect changes in patterns of association of cellular materials was afforded by studies of the action of trypsin. Buffered solutions of this enzyme rapidly cause disintegration of chromosomes and other cellular structures. By resolving the process of tryptic digestion into its component phases—first treating with an aqueous solution of trypsin, then with water, then with a solution of electrolytes, and again with water—it was determined that the enzyme itself causes no disintegration, but that, in the presence of electrolytes, the partially degraded nucleoproteins acquire gel-like properties so that they are able to swell enormously.[14, 15, 23]

Since irradiation of living cells may produce swelling of nuclei and nucleoli, and reversal of mitotic phase (for example, assumption of an interphaselike aspect by early prophase chromosomes), it seemed probable that such changes might be due to alterations in the colloidal properties of cellular materials. Experiments were undertaken, accordingly, to determine whether the capacity of cells to swell when exposed to the trypsin-water-buffer-water sequence of reagents is influenced by exposure to X rays. Salivary-gland cells of *Drosophila melanogaster* were chosen for these tests because they had been used in the aforementioned studies in which the conditions of assay had been established. The larvae were irradiated; the salivary glands were removed at designated

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* Gilbert *et al.*[9] have reported that the products resulting from the reaction of desoxyribonucleohistone with nitrogen mustard are partially resistant to the action of desoxyribonuclease; Taylor *et al.*,[39] however, noted that DNA depolymerized by X rays was hydrolyzed by desoxyribonuclease at the same rate and to the same extent as nonirradiated DNA.

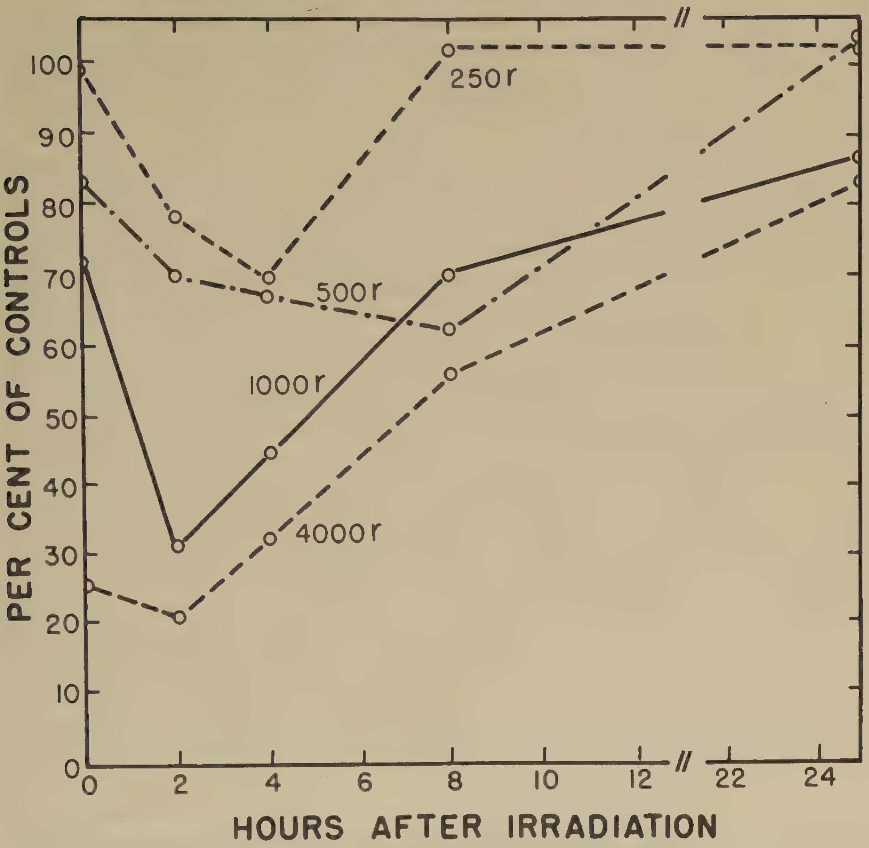

FIGURE 1. Modification, following X-ray treatment of larvae of *Drosophila melanogaster*, of capacity of salivary-gland cells to swell when exposed to the trypsin-water-buffer-water sequence of reagents.

intervals thereafter; fixed in 45 per cent acetic acid; rinsed thoroughly in water; and then subjected to the trypsin-water-buffer-water test.

An extensive series of experiments, involving measurements over a 24-hour period after irradiation, is represented in FIGURE 1. It will be seen from the data presented there that the capacity of the cells to swell depends on the X-ray dose and the time interval after treatment. Minimum values are reached within a few hours, and the capacity to swell then gradually returns to that of the nonirradiated controls. In supplementary experiments, efforts were made to determine more precisely the response during the first few hours. The preliminary data suggest that in some cases there is an initial increase in the swelling capacity, which is shortly followed by a decrease. Our previous studies showed that swelling could be inhibited or retarded if the nucleoproteins were degraded by removal of nucleic acids from the cells. The results of the experiments described above also suggest that swelling can be modified by the degradational action of ionizing radiations on cellular nucleoproteins, but there is no indication that this involves removal of nucleic acids. The swelling of nuclei and nucleoli that occurs in living cells is not dependent, of course, on tryptic digestion, but is explicable in the light of the information afforded by such studies on the basis of water uptake resulting from partial degradation of structural nucleoproteins.

Production of chromosomal aberrations by ribonuclease. The action of X rays in modifying the gel-like properties of cellular nucleoproteins suggested that such changes might be involved in the production of chromosomal aberrations. If so, could these structural modifications be induced by agents capable of degrading specific nucleic acids and proteins, namely, nucleases and proteases? In the first experiments designed to test this possibility, onion roots were grown in solutions of a purified, protease-free, crystalline ribonuclease. This enzyme was chosen because of its low molecular weight, which is about 13,000, and because its substrate, which is RNA, is present in both cell nucleus and cytosome.

The effect of the enzyme solutions on cells of the meristematic region of the roots was revealed by the production of fragmented, adherent, or excessively contracted chromosomes, and by the occurrence of aneuploid and polyploid chromosome complexes, tripolar and multipolar spindles, and binucleate and multinucleate cells. Photographs of such ribonuclease-induced chromosomal aberrations and mitotic abnormalities have been published elsewhere.[20, 21]

The course of the reaction initiated by the enzyme molecules, which eventuates in the production of aberrations in the onion root-tip cells, remains unknown. Conceivably, the presence of a foreign protein, such as ribonuclease, might trigger a number of reactions apart from the action of the molecules on RNA-containing substrates. One line of evidence suggests, however, that some direct action on RNA may be involved. The course of production of abnormalities, which proceeds from the periphery across the root, parallels the course of movement of the enzyme, which is revealed by reduction of stainability of the cells with pyronin. A detailed analysis of the production of aberrations by ribonuclease is presented in another publication.[16]

These studies thus focus attention on the essential role of RNA and ribonucleoproteins in determining chromosome form and distribution in meristematic cells. Adhesion of chromosomes, swelling of nuclei and nucleoli, reversal of mitotic phase (including such localized changes in refractive index as were seen in irradiated chromosomes discussed elsewhere in this monograph by Doctors Zirkle and Bloom), and impairment of mitotic mechanisms may be explained, in the light of the information assembled here, in terms of changes in the gel-like properties of constituent nucleoproteins.

In attempting to visualize a cellular reaction system responsible for the production of mitotic abnormalities, it should be kept in mind that ribonucleoproteins are essential structural components of cytoplasmic bodies that serve as sites for many enzymes. Structural degradation might thus lead to functional impairment and, conversely, functional impairment to structural degradation. In approaching the problem from this point of view, experiments were recently initiated using solutions of cytochrome-c in treatment of onion roots, and this enzyme also was effective in shortening chromosomes, impairing mitotic mechanisms, and producing polyploid chromosome complexes. The nature of the reaction involved is currently under investigation.

Since it was found that ribonuclease produced, in living cells, the types of aberrations that have been designated as "physiological" when induced by ionizing radiations, it remained to be determined whether this enzyme could also simulate radiations in the production of "structural" derangements. Adult flies of *Drosophila melanogaster* were exposed to an aerosol of ribonuclease, using equipment and procedures that had proved effective in studies with nitrogen mustard, but no induced translocations between the second and third chromosomes were found among 1500 spermatozoa tested. Chemical assay demonstrated that production of the aerosol did not inactivate the enzyme to an appreciable extent, so that the negative results indicate either that the enzyme fails to reach the spermatozoa, or that it is unable to break the chromosomes. The aerosol had no significant toxic effect on the flies, since survival was over 90 per cent in all treatments. In related tests on *Bacillus megaterium*, Doctor W. Szybalski found that high concentrations of the enzyme (10 mg. per ml. of agar substrate) did not inhibit growth or division.

Any conclusions drawn from these preliminary data, regarding "physiological" or "structural" changes induced by radition must be tentative, but if further studies confirm the observations presented above, some progress will have been made in distinguishing between these two classes of aberrations in terms of the cellular constituents involved.

The effect of ionizing radiation on the viscosity of gels of nuclear origin. In the light of the foregoing observations that chromosomal nucleoproteins could be altered *in situ* by X rays, it seemed desirable to determine whether the pattern of organization could also be modified by irradiation after the chromosomal materials had been extracted from the cell. Such studies were undertaken with the realization that inferences about biological properties, when derived from extracted materials, are subject to the criticism that the materials have been altered in the course of extraction. In an effort to minimize such alteration, aqueous extracts were used. These extracts afford the closest approximation now known to desoxyribonucleoproteins in their native state.[5, 34]

When isolated calf thymus nuclei are placed in a small volume of distilled water, they first agglutinate and then form a rigid gel. Upon addition of more water, the volume of the gel increases with a concomitant loss of rigidity. Such hydration may be continued until the gel is completely dissipated, leaving a solution of nuclear materials. These gels (and solutions) are extremely unstable, becoming markedly depolymerized—as manifested by liquefaction, loss of viscosity, and the appearance of low molecular weight nucleoprotein fragments—when stored at 5° C. for 24 hours.[6] The instability of these nucleoprotein systems, as compared with the stability of protein-free sodium desoxyribonucleate, might form a basis for the difference between the radiation resistance of DNA[30, 39] and the radiation sensitivity of biological systems.[25] Studies were therefore made of the effect of X rays on these nuclear gels.

The phenomenon chosen for observation was the structural or anomalous viscosity of the nuclear gels, which can be attributed to the networklike structure resulting from interaction of particles. Such structure depends on the

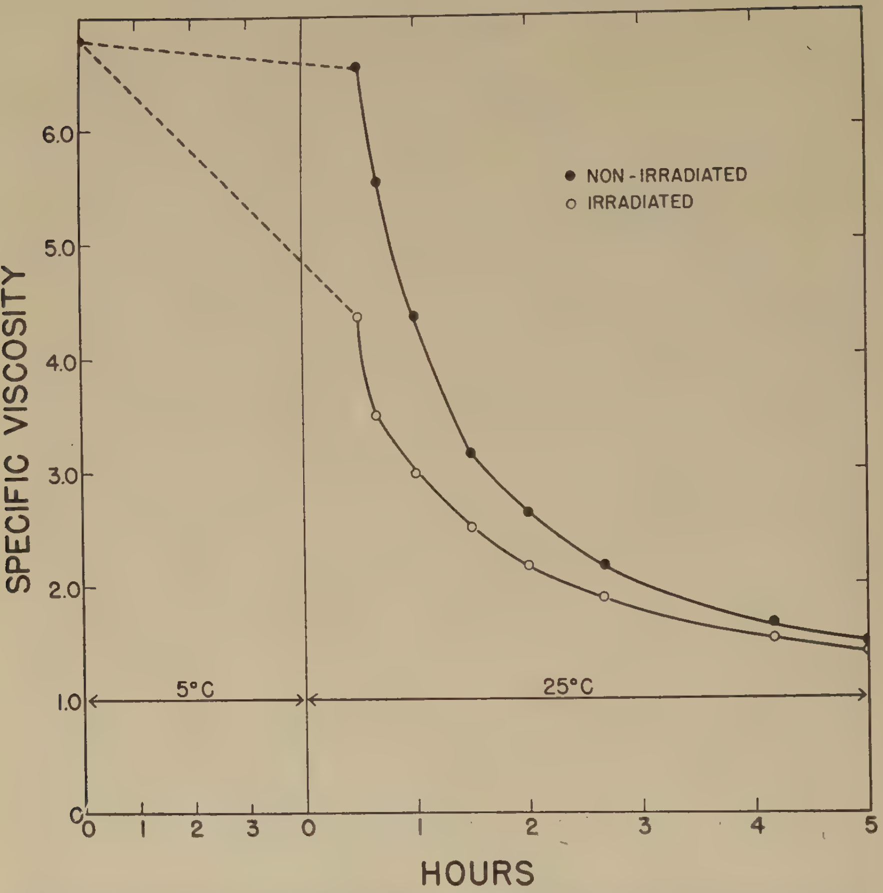

FIGURE 2. The effect of X radiation on the viscosity of nucleoprotein gels. Gels contained 0.068 per cent DNA. After a dose of 1000 r., control and irradiated samples were stored at 5° C. for four hours, then placed at 25° C. and their viscosities measured at intervals.

size, asymmetry, and charge of the particles, and on ionic environment, concentration, and many other factors.[2, 11]

Gels of calf thymus nuclei* were exposed to 1000 r. of X rays, and their viscosities determined at intervals at 25° C. after various periods of storage at 2° to 5° C. As can be seen in FIGURE 2, the viscosities of the gels immediately after irradiation were found to be identical with those of the nonirradiated controls. After four hours at 2° to 5° C. there was an appreciable drop in the viscosities of the irradiated samples. Further storage at 25° C. resulted in additional loss of viscosity in the irradiated gels and a decrease in the viscosity of the nonirradiated ones. The loss of viscosity with time at 25° C., after preliminary storage at 2° to 5° C., follows the same course in both irradiated and nonirradiated gels: the irradiation apparently produces an acceleration of the spontaneous loss of viscosity occurring at 5° C. A major portion of this effect is expressed in four to six hours, though continuing effects have been observed up to 18 hours.

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* Anderson[4] has recently reported that crude, fresh thymus extracts in 1.0 M NaCl are extremely sensitive to X ray doses of as little as 50 r. Shooter[31] has suggested that Anderson's[3] preparations have a high viscosity because they exist in the form of aggregates or gels. If so, Anderson's preparations in 1.0 M NaCl would be very similar to the aqueous nuclear gels that are the subject of the present report.

The spontaneous loss of viscosity of nuclear gels occurring at 5° C. involves a cross-sectional fragmentation of the desoxyribonucleoprotein molecule, as evidenced by the formation of low molecular weight desoxyribonucleoprotein fragments, with a marked decrease in the viscosity of the DNA component.[6] In an effort to determine whether the loss of viscosity induced by irradiation also involves depolymerization of the DNA component, the viscosity of nucleoprotein gels, irradiated and stored in water, was measured either in water or after transfer to 1.0 M NaCl. In the latter case, the constituent nucleic acids and proteins are dissociated, and the viscosity measured is that of the DNA.[26, 34, 35] The results of these measurements are shown in TABLE 1. It can be seen that the magnitude of the radiation effect on the viscosity of the nucleoprotein gel in water is a function of the radiation dose. Radiation does not appreciably alter the viscosity of the DNA component, however, as seen from the measurements in NaCl. The data in TABLE 2 show that the spontaneous decrease in viscosity observed at 25° C. does involve some degradation of the DNA component.

In a further attempt to elucidate the nature of the radiation sensitivity of the naturally occurring nucleoproteins, artificial nucleoproteins of DNA and crystalline bovine serum albumin were made at pH 6.5, and their sensitivity to spontaneous and X-ray degradation was studied. The results are illustrated

TABLE 1

EFFECT OF X RAYS ON THE VISCOSITY OF NUCLEOPROTEIN GELS

Dose	0	250 r.	500 r.	1000r.
Sample	Specific viscosity			
0.3 mgm. DNA per ml.;* viscosity measured in water	10.6	7.64	5.96	4.13
1.2 mgm. DNA per ml.;* viscosity measured in 1.0 M NaCl	2.65	2.80	2.61	2.54

* Concentrations are expressed as amounts of DNA (the gels also contained an equal amount of protein). All samples were irradiated at a concentration of 1.5 mg. DNA per ml., and subsequently diluted for measurement, after storage at 2° to 5° C. for four to six hours.

TABLE 2

EFFECT OF STORAGE ON THE VISCOSITY OF NUCLEOPROTEIN GELS

Sample contained 1.2 mg. DNA per ml.	Specific viscosity		
	Original sample	Aged for 4 hours	
		2°–5° C.	25° C.
Viscosity measured in water	120.0	—	35.0
Viscosity measured in 1.0 M NaCl	2.65	2.61	1.88

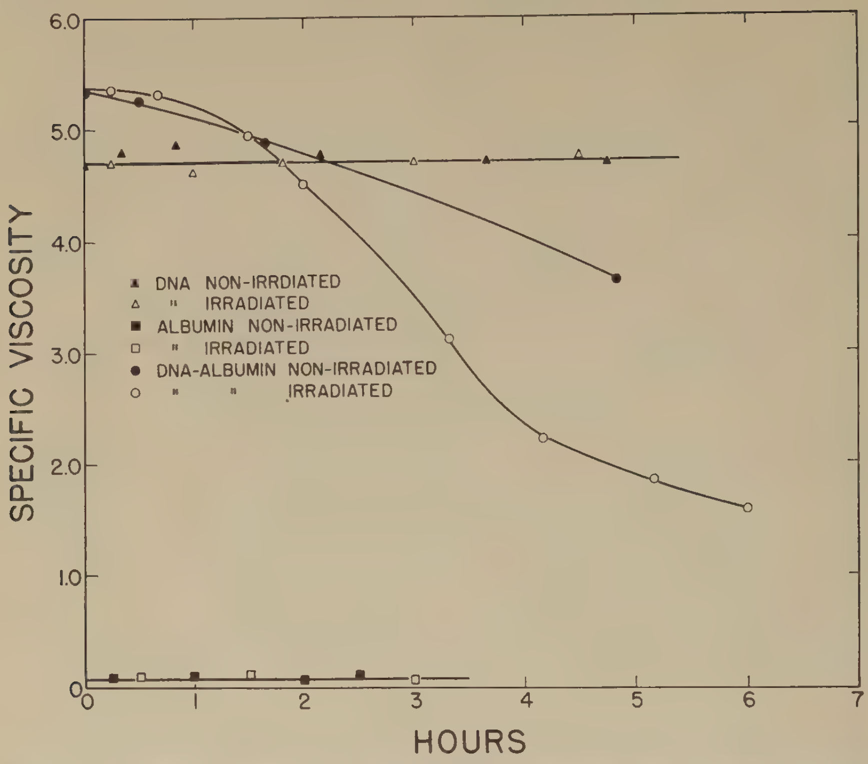

FIGURE 3. The effect of X radiation on the viscosity of DNA, bovine serum albumin, and an artificial nucleoprotein of DNA and albumin. Concentration of solutions, 0.1 per cent DNA and/or albumin. After a dose of 1000 r., the viscosities of both irradiated and nonirradiated samples were measured intermittently at 25° C., without previous storage at 5° C.

in FIGURE 3. It is apparent that the viscosity of the nucleoprotein complex is originally greater than the sum of the viscosities of the individual components,* but decreases when the complex is stored at 25° C. This decrease is enhanced by exposure to 1000 r. of X rays; the radiation-induced decrement in the viscosity becomes apparent after 90 minutes at 25° C., and is completely expressed at 180 minutes, after which it follows the decrease of the nonirradiated control. No change is observed in the viscosity of serum albumin or DNA under these conditions.

These experiments suggested that the radiation sensitivity of the nuclear gels might be related to their protein component. Studies of the effect of trypsin on the viscosity of these systems were therefore undertaken. The experiments are still in a preliminary state, but the results to date indicate that trypsin reduces the viscosity of the nuclear gels, the decrease being approximately proportional to the concentration of trypsin used (FIGURE 4).

Another aspect of these studies dealt with the effects of urea. Urea has been shown to act on groups capable of forming intermolecular associations.[8, 10, 11, 33]

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* This observation contrasts with that of Greenstein and Jenrette,[10] who found that the addition of proteins to solutions of DNA produced a marked drop in viscosity. The reason for the discrepancy is not known.

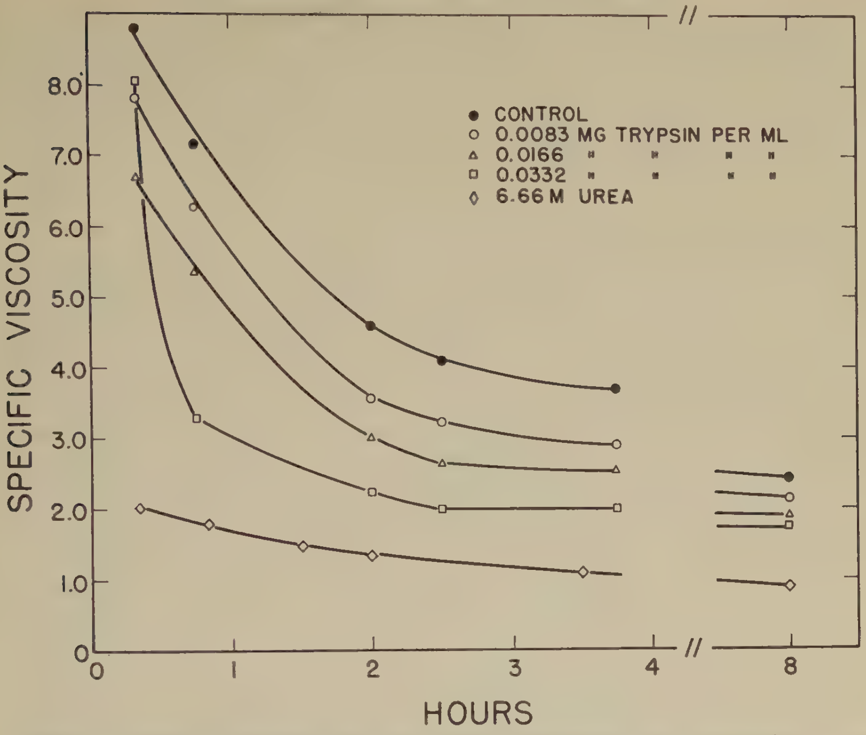

FIGURE 4. The effect of trypsin and of urea on the viscosity of nucleoprotein gels. Gels contained 0.07 per cent DNA. All measurements were made with an applied pressure of 15.0 cm. H_2O.

Nuclear gels were therefore observed viscosimetrically in the presence of 6.6 M urea (FIGURE 4). There was a nearly complete abolition of the unstable, anomalous portion of the viscosity of the gel, providing circumstantial evidence that the structural viscosity of the nuclear gels is attributable to secondary molecular associations.

The data that have been presented suggest that the radiation sensitivity of the nuclear gels is a function of the capacity of the nucleoprotein complex to form intermolecular associations. This suggestion, of course, does not represent a complete explanation of the phenomenon. These data, however, coupled with the consistently shown radiation resistance of protein-free DNA, make it reasonable to assume that the biological effects of ionizing radiation do not involve the direct degradation of DNA. It is considered highly probable, on the contrary, that the biological and genetic effects of ionizing radiations are a function of the nucleoprotein complex, and not of any single constituent thereof.

The effect of X rays on the hydration of isolated nuclei. In another series of experiments, a suspension of isolated nuclei in 0.14 M NaCl was exposed to 1000 r. of X rays, centrifuged, and resuspended in water. It was observed that the capacity of these nuclei to swell when placed in water was markedly reduced. In this case, the effect was immediate, and it decreased with time after irradiation. If a sufficient volume of water was added to permit the formation of a gel, rather than a mere swelling of the nuclei, there was an immediately apparent reduction in viscosity as compared with the nonirradiated controls. This result contrasts with the situation previously described, in which the radiation effects were not expressed immediately.

Summary

Cytochemical methods have been used to study the alterations effected by ionizing radiations in nucleoproteins of dividing cells. It was found that exposure of onion roots to heavy doses of X rays (16,000 r.) did not alter the capacity of the DNA of the meristematic nuclei to bind methyl green or the leucobasic fuchsin of the Feulgen reagent. Irradiation caused some dissociation of the nucleoproteins, however, as evidenced by modifications of stainability of cells of the exposed tissues with the acidic dye, fast green, and the basic dye, pyronin. Differences in response of irradiated and nonirradiated cells to hydrolysis by nucleases also indicated that the pattern of organization of cellular materials was altered by X rays. The capacity of salivary-gland cells of larvea of *Drosophila melanogaster* to swell when treated with an aqueous solution of trypsin, then with water, then with a solution of electrolytes, and finally with water was reduced when the larvae had been exposed to ionizing radiation, demonstrating that structural nucleoproteins were partially degraded by the X rays.

Meristematic cells of onion root tips grown in solutions of ribonuclease showed chromosomal aberrations and mitotic abnormalities similar to those induced by ionizing radiations. In contrast to these ribonuclease-induced "physiological" changes, no ribonuclease-induced "structural" changes were found in a study of induced translocations in *Drosophila.*

The structural viscosity of hydrated nuclear gels was destroyed by treatment with low doses of X rays (250 r.). This loss differed from the spontaneously occurring loss in viscosity. Whereas the latter loss involved cross-sectional fragmentation of the desoxyribonucleoprotein phase, as manifested by the formation of low molecular weight desoxyribonucleoprotein fragments with a marked decrease in the viscosity of the DNA component, the former did not appreciably alter the viscosity of this constituent. Radiation of isolated thymus nuclei was found to reduce their capacity to hydrate.

Just as no single component is the principal structural entity of chromosomes, no single component appears to be the determinant in the response of cells to X radiation. It appears to be the complex nucleoprotein that is altered. Changes, however small, in any of the constituent parts will modify the pattern of organization of the whole complex.

Acknowledgments

The studies summarized in this report have been carried on intermittently since 1948, with the assistance of several individuals to whom we wish to express our sincere thanks. We are indebted to Jessie P. Snyder, Ann J. Swicker, and Marjorie R. Lennihan for painstakingly collecting the extensive data on staining reactions of irradiated cells, and to Mary E. Rowan for making the measurements on trypsin-induced swelling. We are indebted to Nirmal K. Das, Gloria R. Kersh, and Chitra Ghosh for assistance in analyzing the enzyme-induced aberrations, and to Graydon Vanderbilt for assistance with the spectrophotometric determinations. Finally, we wish to acknowledge our indebtedness to Helen Gay for considerable help in organizing some of the experiments and in checking the results.

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TRACK EFFECT IN IOCHEMISTRY* OF AQUEOUS SOLUTIONS

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The effect of the heterogeneous distribution of the initial energetic states on the iochemical reaction kinetics was first discussed theoretically by Lea.[1, 2] Lea used, for this purpose, a "cylindrical" model, in which ensembles of H and OH were distributed initially according to cylindrical symmetrical Gaussian functions, the one representing H reaching farther into the fluid than that representing OH. An approximate kinetic equation for this model was obtained by ignoring the effect of the reactions on the relative distribution of the diffusing radicals. Samuel and Magee[3] extended this treatment by adding a spherical model, by means of which the reactions in the secondary tracks[4, 5, 6, 7] were taken into account. The relative distribution of the two radicals was assumed to be the same in this model.

Both of these works were limited to the reactions in water itself. To develop the subject further, the following calculations were carried out on Samuel and Magee's model, modified by having a single solute (S) in the medium. Besides the simplifications already mentioned, the model also ignores (1) the difference between the radicals, both as regards the diffusion rates and the rate constants which define their interactions with each other and with the solute; (2) secondary reactions of the radicals with the primary reaction products. If the radical is called R, the only reactions considered are, therefore:

$$R + R \xrightarrow{(\alpha)} R_2 \qquad (1)$$

$$R + S \xrightarrow{(\beta)} RS \qquad (2)$$

Half of the molecules R_2 may be assumed to be H_2O, the other half an even mixture of H_2 and H_2O_2. RS may represent a radical, a mixture of two radicals, or a saturated molecule.

I. *Sphere*

The radicals are distributed initially in an infinite medium according to the spherically symmetrical Gaussian function:

$$n_0 = N_0 e^{-r^2/b^2} / \pi^{3/2} \cdot b^3 \qquad (3)$$

N_0 is the initial number of radicals; b is referred to as the initial "equivalent radius" of the reaction zone.

The change in concentration of the radicals in a spherical shell during the time dt is given by equation 4. This equation is strictly valid only when n is large, which it actually is not. It will, nevertheless, serve our present purpose.

$$4\pi r^2 dr(dn/dt) = Dd(4\pi r^2(dn/dr)) - 4\pi r^2 dr(\alpha n^2 + \beta nC) \qquad (4)$$

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* The word "iochemistry" is used in this paper to describe the branch of radiation chemistry which pertains to ionizing radiations.

D: coefficient of diffusion of radicals (cm^2 sec^{-1}); C: concentration of solute (molecules cm^{-3}); α, β: rate constants of radical-radical and radical-solute reactions respectively (molecules^{-1} cm^3 sec^{-1}); n: concentration of radicals (molecules cm^{-3}) at distance r (cm) from center, at time t (sec).

Since the effect of the reactions on the distribution function is neglected, the distribution will remain Gaussian during the expansion. The spatial distribution of the free radicals at time t can therefore be written:

$$n = \frac{Ne^{-r^2/(4Dt+b^2)}}{\pi^{3/2}(r^2 + 4Dt)^{3/2}} \tag{5}$$

where N represents the total number of free radicals in the whole reaction zone at time t. The equivalent radius of the reaction zone is now: $b_1 = (4\mathrm{D}t + b^2)^{1/2}$. We have

$$dN/dt = \int_0^\infty (\alpha n^2 + \beta nC)4\pi r^2\, dr \tag{6}$$

By introducing the value of n from equation 5 into equation 6:

$$dN/dx = -AN^2/x^{3/2} - BN \tag{7}$$

where $b^2x = 4Dt + b^2$; $A = \alpha/4\mathrm{D}(2\pi)^{3/2}$; $B = \beta Cb^2/4D$. This is a Riccati differential equation, the solution of which can be written:

$$N = \frac{N_0e^{B(1-x)}}{1 + N_0A \displaystyle\int_1^x \frac{e^{B(1-x)}}{x^{3/2}}\, dx} \tag{8}$$

The fractional number of radicals N_s/N_0 which have reacted with the solute, at time t, when the equivalent radius of the reaction zone is b_1, is given by

$$\begin{aligned} N_s/N_0 &= \int_0^t (N/N_0)\beta C\, dt \\ &= B \int_1^{(b_1/b)^2} \frac{dx}{e^{B(x-1)}(1 + 2N_0A) - \dfrac{2N_0A}{x^{1/2}} - 2N_0A(\pi B)^{1/2}(\Phi x^{1/2} - \Phi B^{1/2})e^{Bx}} \end{aligned} \tag{9}$$

Φ Gauss function.

II. *Cylinder*

The radicals are distributed initially in an infinite medium according to the cylindrically symmetrical Gaussian function:

$$n_0 = N_0e^{-r^2/b^2}/\pi b^2 \tag{10}$$

where N_0 is the initial number of radicals per cm of axis of cylinder and b is the initial "equivalent radius" of the reaction zone. The length of the cylinder is assumed to be infinite, as compared with the extent of the expansion. The treatment follows the same pattern as above. The solution is:

$$N_s/N_0 = B\int_1^{(b_1/b)^2} \frac{dx}{e^{B(x-1)} + Ae^{Bx}(E_i(-Bx) - E_i(-B))} \qquad (11)$$

E_i : logarithmic integral.

The spatial distribution of the free radicals at time t is given by:

$$n = \frac{Ne^{-r^2/(4Dt+b^2)}}{\pi(4Dt + b^2)} \qquad (12)$$

where N is the total number of free radicals in the whole reaction zone, per cm. of axis of cylinder and $b_1 = (4Dt + b^2)^{1/2}$ is the equivalent radius of the expanded cylinder. The parameters A and B have the same meaning as above.

III. *Ion Track*

The procedure used in applying these formulae to the problem of an isolated ion track is indicated in FIGURE 1. The small solid circles represent the primary reaction zones, which are assumed to be spherical, symmetrical, all alike, and evenly spaced. Their initial equivalent radius is called b_m and the distance between them is E_0/E, when E is the energy dissipation per cm. of track and E_0 is the average energy consumed per reaction zone. The initial number of radicals in a reaction zone is $N_0^{(m)} = G(R)E_0/100$, where $G(R)$ is the number of radicals produced per 100 ev. of absorbed energy.

In order to calculate the kinetic equation for a track, we follow first the reactions in each zone by the spherical formula, until its equivalent radius is $E_0/2E$. Thereafter, the cylindrical formula is used. In using this formula, the initial equivalent radius of the cylinder is taken to be $E_0/2E$ and the initial number of radicals per cm. of cylinder axis is taken equal to the number of radicals found in all the expanded spherical zones, contained in 1 cm. track, at the time when they meet.

When the initial equivalent radius of the primary reaction zone is $\geqq E_0/2E$, the cylindrical formula is used to begin with. The initial equivalent radius of the cylinder is then taken to be $b^{(m)}$ and the initial number of radicals per cm. of cylinder axis is $G(R)\cdot E/100$.

As a numerical example, the reaction curves of FIGURE 2 were calculated using $\alpha = 10^{-11}$, $b_m = 10^{-7}$ cm, $D = 4.5\cdot 10^{-5}$, $G(R) = 8$. The choice of α will be remarked upon below. The energy consumed per reaction zone was taken to be $E_0 = 100$ ev. The track density is given in terms of ionization processes per μ of length of track, from which quantity the specific energy dissipation in the track was obtained by multiplying it by 32.5×10^4 ev. and 35×10^4 ev., for the low and high density tracks respectively. For the sake of convenience, the concentration C is given in the figure in terms of Mole/L. The ratio β/α is represented by $\beta/\alpha = p$.

We have so far assumed that the radiation intensity is so low that the effect of overlapping of the reaction zones from different tracks can be neglected. In order to establish the position of the transition region between negligible and complete overlapping for the model, the kinetic equation is derived for the latter case by assuming that the radicals are produced uniformly

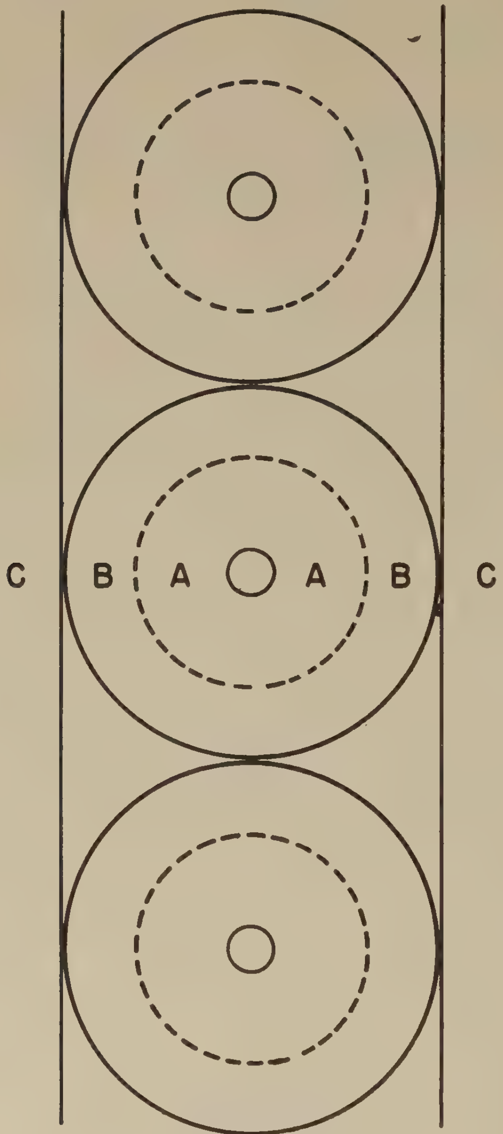

FIGURE 1

throughout the medium. If qI(I: dosage rate in r sec^{-1}) represents the rate of production of radicals per cm^3 of medium, we have in the stationary state (defined, as before, by the sole occurrence of reactions 1 and 2):

$$qI - 2\alpha[R]^2 - \beta C[R] = 0 \tag{13}$$

$$[R] = [(\beta C/4\alpha)^2 + qI/2\alpha]^{\frac{1}{2}} - \beta C/4\alpha \tag{14}$$

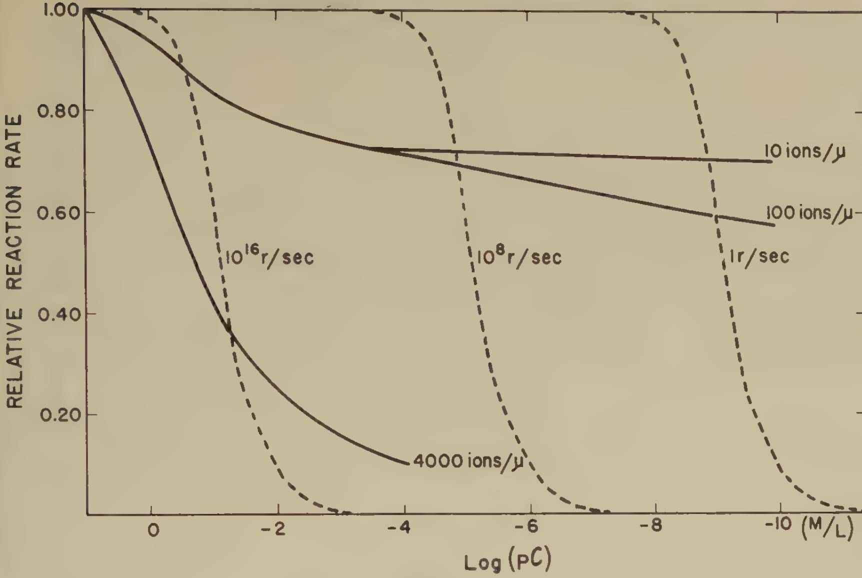

FIGURE 2

The fractional number of radicals which react with the solute is:

$$N_s/N_0 = \frac{[R]\beta C}{qI} = x\left(\left(1 + \frac{2}{x}\right)^{1/2} - 1\right) \tag{15}$$

$$x = (\beta C)^2/4q\alpha I = (pC)^2\alpha/4qI \tag{16}$$

The form of these curves is also shown in FIGURE 2, calculated for different values of I, by using

$$q = (1.6.10^{12}\cdot 32.5\cdot 8)/100 = 4.2\cdot 10^{12}$$

The following discussion deals with the question of the probable values of the rate constants for the interaction of the radicals. In the calculations in his monograph[1], still widely quoted, Lea used the value 4×10^{-10} (molecules^{-1} cm^3 sec^{-1}) for the rate constant for the combination of H and OH (the only reaction considered in this work), which was calculated from the gaskinetic collision number and therefore would be expected to be too high. The value was later[2] revised downward to 10^{-10} on the basis of a theoretical formula of Fowler and Slater.[8] In comparing these values with the rate constant α used in the present work, they should be divided by 2, to take into account the particular ways in which rate constants are defined when similar and dissimilar particles interact. The value of α used by Samuel and Magee[3] was $\alpha = 10^{-11}$.

Let us consider certain extraneous experimental evidence which bears on this question. Formally, the rate constant for the interaction of two molecular species dissolved in a fluid medium can be written as the product of the specific encounter rate Z and a factor p, which represents the probability that an encounter between two solute molecules leads to reaction. Although various attempts have been made to calculate Z, no rigorous theory is at present available. Certain lines of reasoning[8, 9] lead to an expression of the form:

$$Z = A(\sigma_1 + \sigma_2) \cdot (D_1 + D_2) \tag{17}$$

where D_1 and D_2 are the coefficients of diffusion of the two molecular species, σ_1 and σ_2, their collisional radii and A is a constant. Fowler and Slater,[8] on the basis of a rather elaborate analysis carried out on the assumption that the molecular diameters of solute and solvent were equal, arrived at the value $A = 30$. Other theories lead to considerably smaller values.[9]

Comparison with experiment can be made by using recent values for the rate constant for the reaction $I + I \overset{(\alpha)}{=} I_2$ in hexane and carbontetrachloride,[10, 11] *viz.*:

In hexane: $\alpha = 3.7 \times 10^{-11}$ (See reference 10)

$\alpha = 1.8 \times 10^{-11}$ (See reference 11)

Mean: $\alpha = 2.7 \times 10^{-11}$

In carbontetrachloride: $\alpha = 1.2 \times 10^{-11}$ (See reference 11).

Since the activation energy of the reaction between these two atoms is presumably nil, we may tentatively take $p = 1$. The theoretical value of α is therefore $\alpha = \frac{1}{2} \cdot Z = 2A\sigma D$. The collisional diameter of the iodine atom may be set at $2\sigma = 4 \times 10^{-8}$ cm. The coefficients of diffusion of atomic iodine in the organic solvents are not known experimentally, but from the values of D for molecular iodine in hexane ($D = 2.4 \times 10^{-5}$) and in carbontetrachloride ($D = 1.8 \times 10^{-5}$), the values of D for atomic iodine may be estimated to be $D = 4 \times 10^{-5}$ and $D = 2 \times 10^{-5}$ respectively.

The value of A is accordingly calculated to be: Hexane $A = 17$. Carbontetrachloride $A = 15$.

We may use the mean of these two values $A = 16$ to calculate the mean rate constant for the combination of H and OH in water. The value of p is assumed to be unity. The mean value for the diffusion coefficients of the radicals may be set at $D = 4.5 \times 10^{-5}$, the mean value of their diameters $2\sigma = 2 \times 10^{-8}$. We obtain then $\alpha = 1.4 \times 10^{-11}$. The value $\alpha = 10^{-11}$ used by Samuel and Magee[3] appears therefore to be about as good a choice as can be made at present. It was used in calculating the graphs of FIGURE 2.

If Stokes-Einstein equation is taken to apply with respect to the relation of D to σ, equation 17 can be written:

$$Z = A_1 \frac{(\sigma_1 + \sigma_2)^2}{\sigma_1 \cdot \sigma_2} \tag{18}$$

where A_1 is a constant. This expression is independent of the absolute values of σ_1 and σ_2 and varies relatively slowly with changes in σ_2/σ_1, so long as this ratio is not too far from unity. Applied to the interaction of the radicals in water, the assumption of the present calculation that the rate is independent of kind of radical may be expected therefore to be approximately correct, provided all the reactions are characterized by values of $p = 1$.

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THE EFFECT OF IONIZING RADIATIONS ON SYSTEMS OF BIOLOGICAL IMPORTANCE*

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A knowledge of radiation chemistry is essential to the radiobiologist for the elucidation of the changes produced in the cell by ionizing radiations. The radiobiologist must apply this knowledge with caution, however, bearing always in mind that the effect of ionizing radiations varies greatly not only quantitatively but also qualitatively when environment and intensity are changed. Moreover, effects obtained on biological systems *in vitro* may not be found *in vivo*, since to the initial effect produced by ionizing radiations on a particular system there may be added effects produced by the radiation products themselves. The interest of the biologist is narrowed to the effect of ionizing radiations in aqueous solutions saturated with oxygen because the living cell contains about 80 per cent of oxygen-saturated water (except the so-called anaerobic cells, and animals living on the lower depths of the sea). That is why the direct-hit theory was never fruitful, and kept radiobiology stagnant, since from simple statistical consideration, the probability of direct hit between water and the ionizing track is at least 10^4 times as much as a hit to a biologically important substance.

I shall therefore confine myself to a discussion of work performed in my laboratory on the effect of moderate doses of X rays upon systems of biological importance dissolved in aqueous aerated solutions.

Oxidation Reductions Produced by Ionizing Radiations

It is generally agreed that, on irradiation of water by X rays, gamma rays, or electrons, there is formation of the oxidizing radical OH and of the reducing radical H atom. If no other reactions occurred, irradiation of aqueous solutions would produce indiscriminate oxidation and reduction processes by the simultaneous action of these radicals upon the solutes present in water. The numerous investigations on the effect of ionizing radiations on oxidation-reduction systems have shown that, contrary to this assumption, oxidation is the dominant feature, and reduction is observed only on systems with an E_0 above + 0.8 volt.[1] The lack of reducing ability may be explained by the rapid recombination of H atoms to give the sluggish, almost unreactive H_2 molecule.

Irradiation of aerated water by these radiations yields H_2O_2, which can be explained by the step-by-step reduction of molecular oxygen by the hydrogen atoms:

$$H + O_2 = O_2H;$$

$$O_2H + H = H_2O_2.$$

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We have thus in the presence of oxygen the formation of one more oxidizing agent, the perhydroxyl radical O_2H which, in the absence of oxidizable substrates in the water solution, will be converted into the stable reduction product H_2O_2. Although the oxidation-reduction potential of H_2O_2 is still high enough ($E_0 = +0.682$ volt) to produce oxidations, the system is, in general, sluggish in the absence of suitable catalysts such as Fe^{++} ions which give the active oxidizing radical OH. All the reduction processes found on irradiation occur in inorganic systems of no importance to the biologist, because the oxidation-reduction systems of biological significance have E_0 values much below those of the oxidizing radicals. It may be said that ionizing radiations produce mostly oxidations.

It is known that sulfhydryl compounds, in the form of nonprotein —SH compounds and —SH proteins, are universally present in the living cell and play an important role in its activities.[2] These compounds are readily oxidized by atmospheric oxygen in the presence of trace amounts of heavy metals,[3, 4] an oxidation which is reversible, provided a suitable mediator is present:

$$2RSH + O_2 \rightleftharpoons RSSR + H_2O_2 .$$

Glutathione, the nonprotein —SH tripeptide, is a sluggish oxidation-reduction system with strong reducing power. Because of this reducing power, it keeps the —SH proteins in the reduced state wherever they have been oxidized into the disulfide state.[5] Under the influence of ionizing radiations, it is readily oxidized with a radiochemical yield G (G = number of molecules reacting per 100 e.v.) of 10.7 when irradiated in aerated water solutions of pH 7.[6] If we postulate that the oxidation of glutathione (GSH) takes place in two steps with the intermediate formation of a half-oxidized free radical $G\dot{S}$, the oxidation of glutathione on irradiation of aqueous aerated solutions can be formulated as follows:

$$GSH + OH \rightarrow R\dot{S} + H_2O \tag{1}$$

$$GSH + O_2H \rightarrow G\dot{S} + H_2O_2 \tag{2}$$

$$G\dot{S} + G\dot{S} \rightarrow GSSG \tag{3}$$

$$GSH + H_2O_2 \rightarrow \dot{G}S + H_2O + OH \tag{4}$$

$$GSH + OH \rightarrow \dot{G}S + H_2O \tag{5}$$

$$G\dot{S} + G\dot{S} \rightarrow GSSG \tag{6}$$

There are thus 12 molecules of glutathione capable of being oxidized by 100 e.v. Since 10.7 was the value found on X irradiation, the efficiency of the oxidation process would be 89 per cent. In the absence of oxygen, when no O_2H radicals are being formed on X irradiation of water, the G value fell to 3.5, a demonstration that under those conditions only the OH radical was active.

The increased oxidation of glutathione on X irradiation of aerated aqueous solutions was undoubtedly due to oxidation by the O_2H radical and by H_2O_2.

TABLE 1

OXIDATION OF GLUTATHIONE BY X IRRADIATION OF AQUEOUS SOLUTIONS
2×10^{-4} *M* glutathione dissolved in 0.01 M phosphate; buffer pH 7.0;
X-ray dose, 5,000 r.

Exp. conditions	G
In the presence of O_2	10.7
In the absence of O_2	3.5
In the presence of catalase and O_2	8.6
O_2 and serum albumin	10.7

To distinguish the effect of the first oxidant from the second, small amounts of catalase were added to destroy H_2O_2. In the absence of catalase, the *G* value was 10.7, and in the presence of catalase it was 8.6 (TABLE I). Glutathione is thus oxidized by the three oxidizing agents produced on irradiation of aerated solutions. What has been said for glutathione applies to other non-protein —SH compounds, such as cysteine and coenzyme A. The —SH group of coenzyme A is oxidized with a *G* value of 9.2, similar to that of glutathione. Oxidized glutathione and cystine are not reduced on X irradiation of deaerated aqueous solutions. When large doses of X radiation are used, there will be, of course, other effects besides reversible oxidation of the —SH groups. In fact, Kinsey[7] found destruction of the glutathione molecule on X irradiation with 50,000 to 100,000 r. The efficiency of this process, however, is far below that of oxidation, the *G* value being from 1.2 to 5.7. X irradiation of glutathione and of cysteine (2×10^{-4} *M*) with 48,000 r. oxidized completely the —SH groups of these two substances without affecting the NH_2 groups, a demonstration of the selective action of ionizing radiations. Dale found, on irradiation with larger X-ray doses, H_2S formation a less efficient process, since the *G* value was 0.8.[8] This reaction is, according to Dale, independent of the presence of O_2 and of H_2O_2.

A large number of proteins have —SH groups in their side chains, either in the form of cysteine residues or as conjugate compounds with glutathione. In some proteins, the —SH groups are so distributed along the polypeptide chain as to be easily attacked by mild alkylating and oxidizing agents. They also combine readily with nitroprusside. They may be called *freely reacting* —SH groups. In other proteins, the —SH groups are widely separated so that their oxidation to the —SS— form becomes rather difficult or impossible; or there are, close to the —SH groups, electronegative groups or other steric hindrances which hamper oxidation. These —SH groups may be called *masked* —SH groups. Finally, there are —SH groups which do not become reactive until the protein has been denatured. Of all these —SH groups, only the freely reacting groups are sensitive to ionizing radiations in small doses. This conclusion was drawn from experiments on the effect of ionizing radiations upon —SH enzymes.[9, 10, 11]

Phosphoglyceraldehyde dehydrogenase and adenosinetriphosphatase are proteins with freely reacting —SH groups; moreover the former has one glutathione attached to the protein.[12] On X irradiation with moderate intensities

TABLE 2

INHIBITION OF PHOSPHOGLYCERALDEHYDE DEHYDROGENASE AND ADENOSINE TRIPHOSPHATASE BY X RAYS; REACTIVATION WITH GLUTATHIONE (ADDED AFTER X IRRADIATION)

Enzyme	X ray dose (r)	Inhibition (%)	Reactivation (%)
Phosphoglyceraldehyde dehydrogenase	100	21	Complete
	200	50	62
	300	80	—
	500	94	10
Adenosinetriphosphatase	100	27	97
	500	41	56
	1000	73	22

TABLE 3

YIELDS OF ENZYMES INACTIVATED BY X IRRADIATION

	—SH active groups	Yield, G (100 ev)
Yeast alcohol dehydrogenase	+	3.4
Phosphoglyceraldehyde dehydrogenase	+	2.9
Carboxypeptidase	−	0.55
d-Amino acid oxidase	+	0.31
Ribonuclease	−	0.09
Trypsin	−	0.077
Hexokinase	+	0.033
Lysozyme	−	0.03
Catalase	−	0.009

(100 r.), the inhibition of activity in these two enzymes was completely reversed when glutathione was added after irradiation, a demonstration that inhibition was due *only* to oxidation of the —SH groups. As the irradiation dose increased, the degree of reactivation of the inhibited enzyme decreased, an indication of irreversible damage of the enzyme such as rupture of H bonds, deamination, *etc.* (TABLE 2). D-amino acid oxidase and hexokinase are —SH enzymes containing masked —SH groups, and oxidation is not easily accomplished. As a consequence, they are more resistant to inhibition by ionizing radiations (TABLE 3).

A comparison of the *G* values on irradiation of glutathione and of —SH proteins shows that the first is far more reactive. Hence glutathione, cysteine, and other nonprotein mercaptans will act as protecting agents. Addition of glutathione protected the enzyme activity of —SH enzymes against X irradiation inhibition.

It must be concluded from these experiments that the —SH groups—at least the freely reacting groups—are so sensitive to oxidation by ionizing radiations that they are probably the first groups destroyed on irradiation with small doses. When the molecule has a number of groups susceptible of attack, the —SH groups will be the first to react. When the dose is increased, other groups as well will be destroyed. In cysteine, for example, there is first oxidation of the —SH groups, then deamination, and finally splitting of the molecule.

The problem of the possible role of atomic hydrogen and of H_2O_2 in cell metabolism was studied by irradiation of ferrocytochrome *c* (which, when purified, is not oxidized by molecular oxygen or by H_2O_2) and of ferricytochrome (which is readily reduced by atomic hydrogen). On X irradiation of deaerated solutions of cytochrome *c* there was oxidation to ferricytochrome *c*, as measured by the disappearance of the absorption band at 5500 Å. The *G* value for this oxidation was 1.65; on irradiation in the presence of air, the *G* value was the same. Moreover, addition of catalase had no influence at all on the extent of oxidation. X irradiation of ferricytochrome *c* with doses up to 100,000 r. produced no reduction.[13] These experiments demonstrate that the atomic hydrogen that is produced on X irradiation of water must convert too rapidly to molecular hydrogen to be a powerful reducing agent. Oxidation of ferrocytochrome *c* by X irradiation is reversible, and the changes in the absorption spectrum are abolished on addition of $Na_2S_2O_4$. Oxidation of the Fe^{++} compound by OH radicals is the primary reaction:

$$Fe^{++} + OH \rightarrow Fe^{+++} + OH^-.$$

This oxidation can be performed by chemically-produced OH radicals, namely by addition to ferrocytochrome of $FeSO_4 + H_2O_2$ ($Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + HO + HO^-$).[14]

On irradiation with larger X-ray doses, there is also an attack on the porphyrin molecule and on the protein moiety, as shown on X irradiation of ferricytochrome *c* with 100,000 r. (FIGURE 1). The large absorption band at 4000 Å., the *Soret band*, is decreased, the decrease being proportional to the X-ray dose. There is also an increase of the absorption band at 2800 Å., which is due to the protein moiety.

Hemoglobin, the ferroporphyrin complex of globin and myohemoglobin, the ferroporphyrin complex of myoglobin (the protein moiety of myohemoglobin is commonly called myoglobin) are oxidized by atmospheric oxygen to the ferric state, besides being oxygenated to oxyhemoglobin and oxymyohemoglobin. It is well known that hemoglobin has four Fe^{++}-porphyrins per mole of protein, while myohemoglobin has only one. On X irradiation of oxyhemoglobin there was rapid oxidation to methemoglobin, the extent of oxidation being directly proportional to the X ray-dose (FIGURE 2). This oxidation must take place according to the following reactions:

$$Fe^{++}Mb + OH \rightarrow Fe^{+++}Mb + OH^-;$$

$$Fe^{++}Mb + O_2H \rightarrow Fe^{+++}Mb + O_2H^-$$

$$2\ Fe^{++}Mb + H_2O_2 \rightarrow 2\ Fe^{+++}Mb + 2\ OH^-$$

$$Fe^{++}MbO_2 + HO_2 \rightarrow Fe^{+++}Mb + O_2 + O_2H^-.$$

The *G* value for this oxidation was 0.08, which is rather low when compared to the *G* value for oxidation of ferrocytochrome *c*. On X irradiation of oxyhemoglobin with 60,000 r. there was, besides its oxidation to methemoglobin, a de-

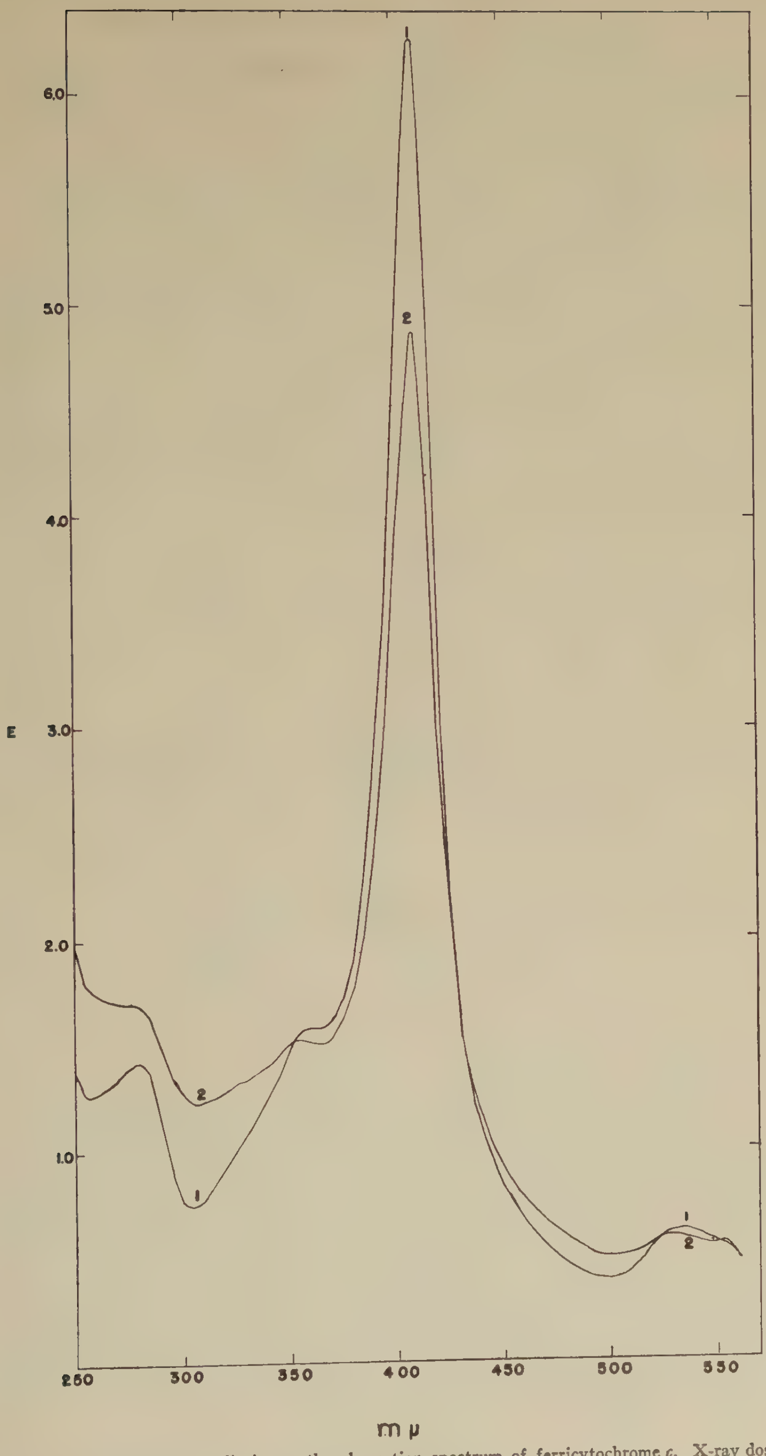


FIGURE 1. Effect of X-irradiation on the absorption spectrum of ferricytochrome *c*. X-ray dose, 100,000 r. Abscissa, wave length in mμ. Ordinate, optical density. (1) Control. (2) X-irradiated.

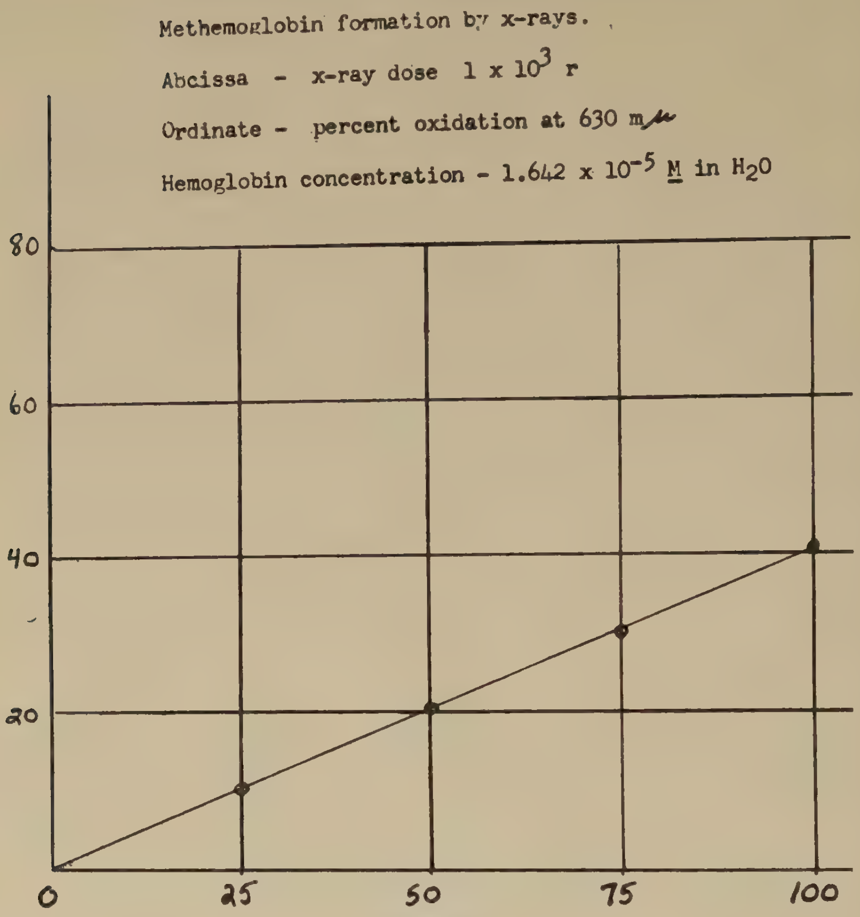


FIGURE 2

crease of the Soret band and a decrease of the absorption band at 2800 Å. corresponding to globin (FIGURE 3).

In TABLE 4 are given the *G* values for the oxidation of some oxidation-reduction systems of biological importance. Contrary to a belief widely held, H_2O_2 takes a negligible part in oxidations produced by ionizing radiations.[14, 15] This finding is easily confirmed by studying the mechanism of H_2O_2 formation by X irradiation of water. The first product formed is the perhydroxyl radical O_2H, which is an efficient oxidizing agent, more powerful than H_2O_2. In the presence of oxidizable substances the O_2H will react immediately and, as a consequence, there will be no possibility for H_2O_2 formation ($O_2H + H \rightarrow O_2H_2$). Hydrogen peroxide will be formed only in the absence of oxidizable substances or when the O_2H radicals are ineffective oxidizing agents.

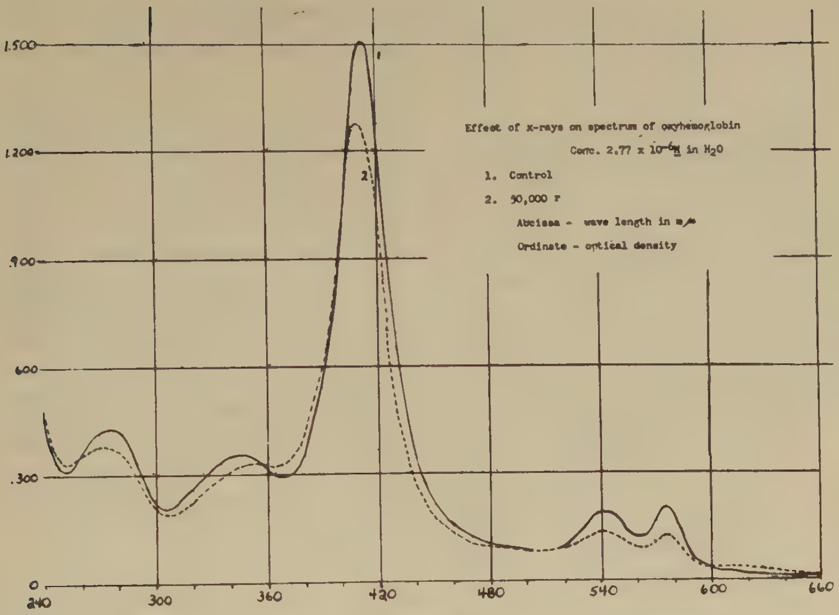


FIGURE 3

TABLE 4

THE EFFICIENCY OF IRRADIATION ON THE OXIDATION OF SOME BIOLOGICALLY IMPORTANT SUBSTANCES. IRRADIATIONS IN AERATED SOLUTIONS

Oxidizable substance	G
Glutathione	10.7
Coenzyme A	9.2
Ascorbic acid	2.1
Cytochrome *c*	1.3
Oxyhemoglobin	0.08
DPNH	1.7
Ethyl alcohol	5.9

Enhancement of Radiation

Most biological oxidations are bivalent, where two electrons are transferred in the oxidation process. According to Michaelis,[16] these bivalent oxidations take place stepwise with the intermediate formation of a half-oxidized free radical. This process was postulated for the reduction of O_2 to H_2O_2, where the intermediate free radical O_2H is first formed. These intermediate free radicals are powerful reactants for oxidations and reductions, and may be able to produce reactions not given by the completely reduced or oxidized parent compound. It is known that diphosphopyridine nucleotide (DPN^+) is not reduced by X irradiation, whereas the reduced compound, DPNH, is oxidized.[17] Swallow[18] found that, on irradiation of deaerated solutions of ethanol plus

DPN^+, there was an increase in the absorption spectrum at 3400 Å., which he interpreted as formation of DPNH. We have repeated Swallow's experiments and have confirmed them. Moreover, we have found that the substance formed is reduced diphosphopyridine nucleotide by testing it enzymatically. Half of the reduced compound was reoxidized by alcohol dehydrogenase and acetaldehyde.[19] Similar results were obtained on X irradiation of lactate, and of isopropyl alcohol plus DPN^+. Lactate was oxidized to pyruvate, isopropyl alcohol to acetone; and DPN^+ was reduced to DPNH. These experiments are coupled oxidation-reductions catalyzed by the free radicals produced on X irradiation of water. The initial process is the oxidation of the oxidizable substrate (for example, lactate) by the free radicals. Here there is formation of the intermediate free radical half-oxidation product $CH_4\dot{C}OHCOO^-$, which will reduce DPN^+ to the half-reduced free radical $DP\dot{N}H$. A second molecule of the half-oxidized lactate completes the reduction of the half-reduced $DP\dot{N}H$:

$$CH_3CHOHCOO^- + OH \rightarrow CH_3\dot{C}OHCOO^- + H_2O;$$

$$CH_3\dot{C}OHCOO^- + DPN^+ \rightarrow DP\dot{N}H + CH_3COCOO^-;$$

$$CH_3CHOHCOO^- + OH \rightarrow CH_3\dot{C}OHCOO^- + H_2O;$$

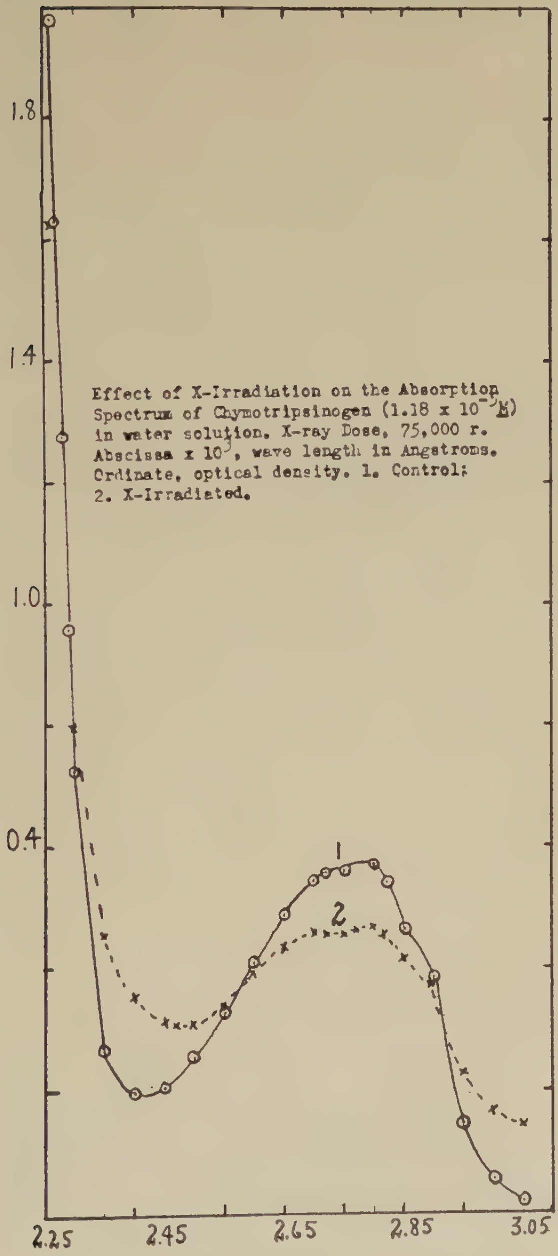
$$CH_3\dot{C}\,OHCOO^- + DP\dot{N}H \rightarrow CH_3COCOO^- + DPNH + H^+.$$

In the presence of ethanol, the free radical would be $CH_3\dot{C}HOH$ and, in the presence of isopropanol, it would be $CH_3\dot{C}OHCH_3$. Since living cells contain a large number of oxidizable substances, it is possible that such reactions may take place on irradiation of living cells. This phenomenon is an enhancement because ionizing radiations alone were unable to reduce DPN^+. My laboratory is now studying the biological importance of these coupled oxidation-reductions which may perhaps be used for the local enhancement of irradiations.

X Irradiation of Proteins and of Amino Acids

We have already shown that proteins are very sensitive to the action of ionizing radiations (TABLES 2 and 3). Enzyme activity was inhibited. It is also known that large doses of ionizing radiations will produce denaturation and, finally, precipitation.[20] In general, irradiation produces an increase in the ultraviolet absorption spectrum which has been attributed to oxidation of the tyrosine residue. This hypothesis has been tested by irradiation of proteins with $\frac{\text{tyrosine}}{\text{tryptophan}}$ ratio lower than one. X irradiation of lysozyme $\left(\frac{\text{tyrosine}}{\text{tryptophan}}\right.$ ratio of 0.6) and of chymotrypsinogen $\frac{\text{tyrosine}}{\text{tryptophan}}$ ratio of 0.45) produced a decrease in the absorption spectrum (FIGURE 4).

The absorption of light at 2800 Å. by proteins is attributed to the aromatic amino acids. Irradiation of tyrosine increased light absorption at this wave length (FIGURE 5), whereas irradiation of tryptophan decreased it (FIGURE 6). In both cases, the changes in light absorption (increase as well as the decrease) were proportional to the radiation dose. These observations confirm the



Effect of X-Irradiation on the Absorption Spectrum of Chymotripsinogen (1.18 x 10^{-5} M) in water solution. X-ray Dose, 75,000 r. Abscissa x 10^{3}, wave length in Angstroms. Ordinate, optical density. 1. Control; 2. X-Irradiated.

FIGURE 4
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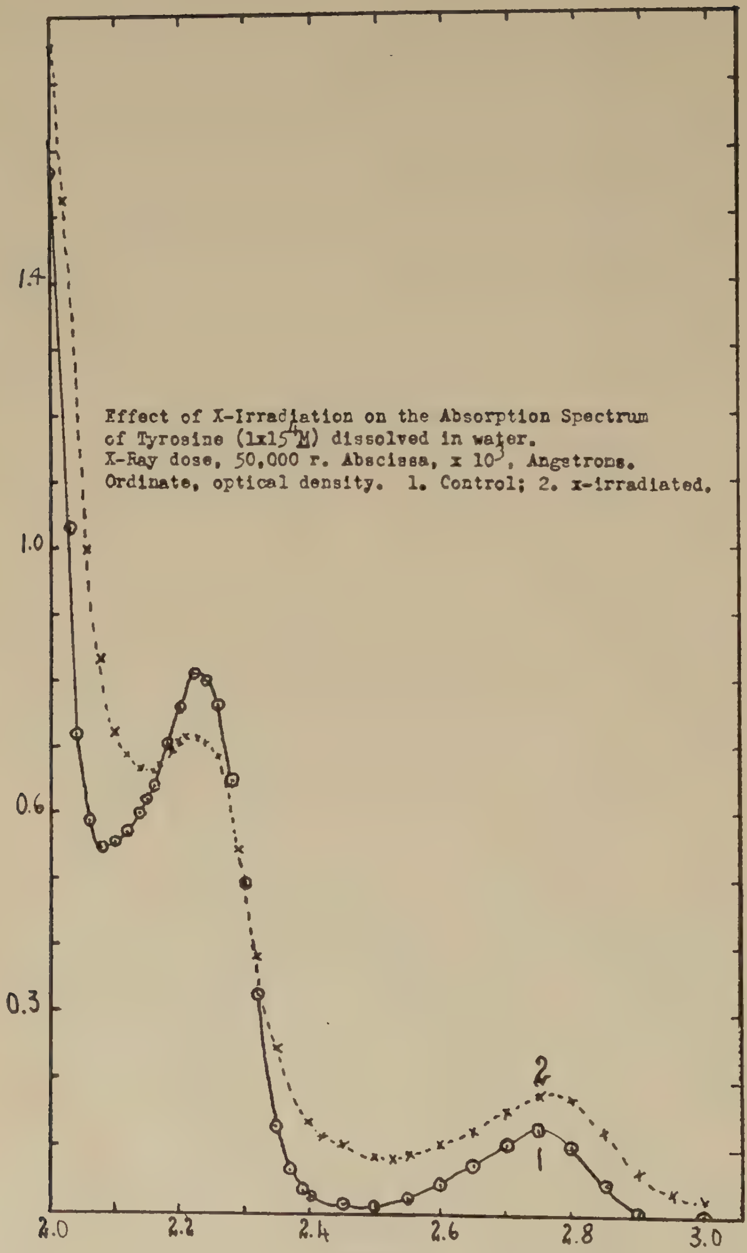
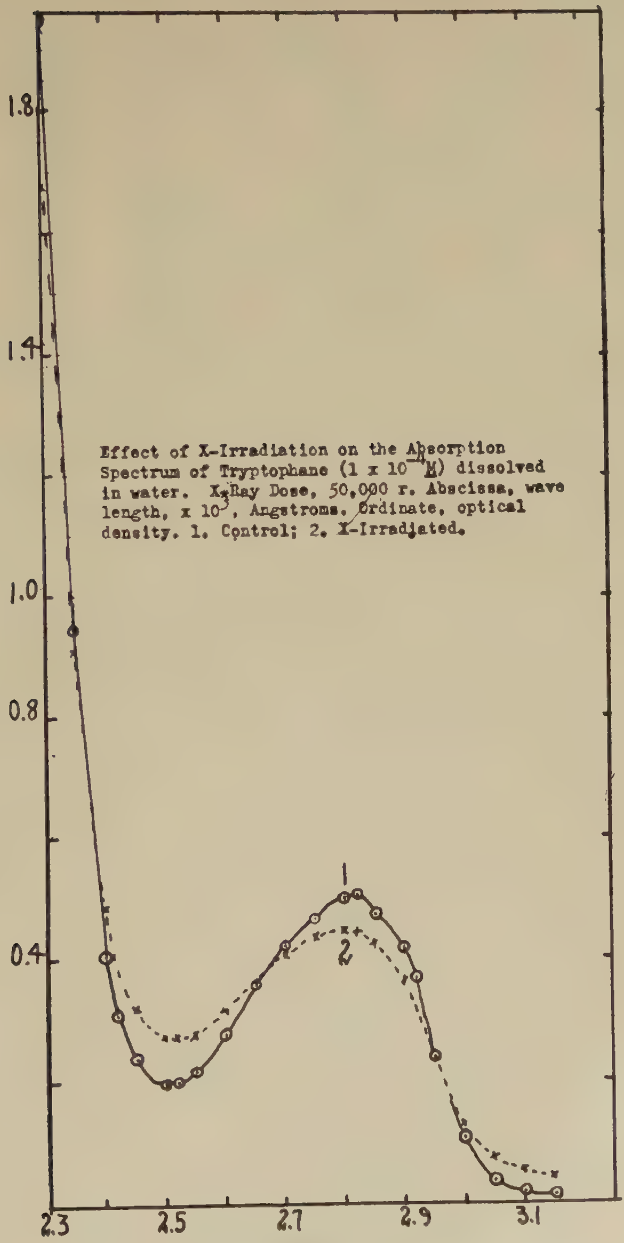


Effect of X-Irradiation on the Absorption Spectrum of Tyrosine (1x15^{4}M) dissolved in water. X-Ray dose, 50,000 r. Abscissa, x 10^{3}, Angstroms. Ordinate, optical density. 1. Control; 2. x-irradiated.

FIGURE 5



Effect of X-Irradiation on the Absorption Spectrum of Tryptophane (1×10^{-4} M) dissolved in water. X-Ray Dose, 50,000 r. Abscissa, wave length, $\times 10^{3}$, Angstroms. Ordinate, optical density. 1. Control; 2. X-Irradiated.

FIGURE 6

TABLE 5

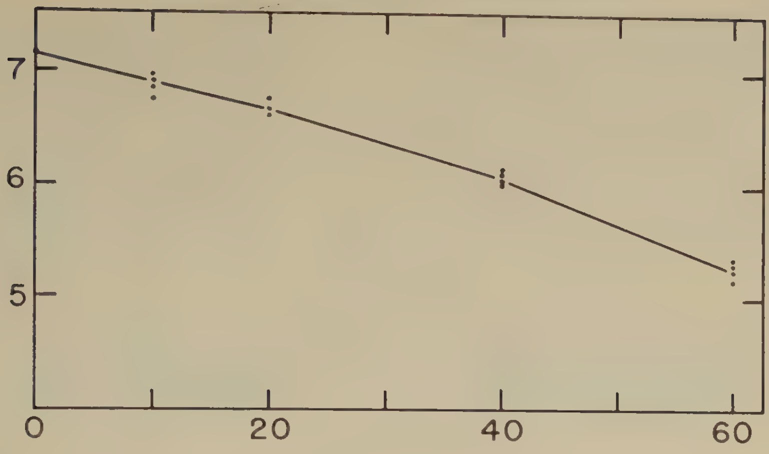
DETERMINATION OF AMINO ACIDS OF SERUM ALBUMIN AFTER X IRRADIATION
Concentration of albumin, 1×10^{-5} *M*, dissolved in water. Figures give g. per 100 g. protein

Amino acids	Control	50,000 r.	75,000 r.
Glycine	1.88	1.72	1.32
Alanine	6.78	5.13	4.41
Valine	5.53	5.54	5.66
Leucine	11.90	10.75	10.96
Isoleucine	2.59	2.51	2.26
Proline	5.12	4.72	4.71
Phyenylalnine	6.37	5.89	5.89
Arginine	6.01	6.30	6.15
Histidine	4.01	4.14	—
Lysine	12.8	10.75	10.10
Aspartic acid	10.6	10.8	10.9
Glutamic acid	17.4	13.1	11.7
Serine	4.42	4.55	4.44
Threonine	5.94	5.11	4.86
Tyrosine	5.15	4.51	4.33

hypothesis that the changes in absorption spectrum found on irradiation of proteins are those seen on irradiation of amino acids.

Irradiation of aqueous solutions of serum albumin with 75,000 r. produced precipitation, which can be avoided by the addition of salts. Irradiation with 50,000 r. produced no precipitation. The irradiated solutions were hydrolyzed with HCl, and the amino acids were determined by paper chromatography. Glycine, alanine, and glutamic acid were decreased by 30 per cent. Lysine, threonine, tyrosine, and isoleucine were decreased by 21, 18, 16, and 13 per cent. Arginine, histidine, aspartic acid, and serine were not affected (TABLE 5). These alterations were also demonstrated by studying the combining power of the serum albumin with anions and cations. X irradiation produced a continuous decrease in the combining power of serum albumin for Orange II (FIGURE 7). It removed from the protein some of the cationic loci, thus reducing the extent of binding of anions. The combination of Cu^{++} with albumin is stronger at pH 4.7.[21] At this pH value, X irradiation with X ray doses up to 60,000 r. had practically no effect on the binding power of albumin to the metal. At pH 5.7 and 6.5, there was definite increase in the number of sites available for combination with copper, as shown by the increased number of copper atoms bound to the protein after X irradiation (TABLE 6). Different results were obtained on X irradiation of the copper-containing pigment, hemocyanine. On irradiation with large doses of X rays, the copper left the protein, as shown by the decrease in the absorption spectrum at 5750 A, which is characteristic of copper complexes with the amino groups of protein (FIGURE 8).

Few changes were observed on the electrophoretic mobility of albumin and globulin solutions in phosphate buffer after X irradiation with 100,000 r. Irradiation with 200,000 r. produced a definite decrease. At pH 3, there was lack of enantiography, both in the control and in the irradiated samples, probably because of pH and conductivity changes across the boundary, as described



Effect of X-Irradiation on the Binding of Anionic Dyes with Bovine Serum Albumin. Buffer, 0.01 M phosphate, pH, 6.84. Albumin, 9.15 x 10^{-8} M. Orange II, 1.113 x 10^{-4} M. Abscissa, x-ray dose in r units x 10^3. Ordinate, number of Orange II molecules combined to one mole of protein.

FIGURE 7

TABLE 6

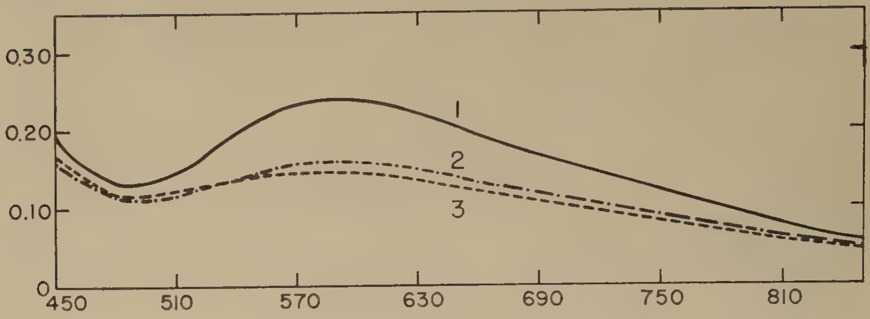
EFFECT OF X IRRADIATION ON THE COMBINING POWER OF BOVINE SERUM ALBUMIN WITH COPPER

Serum albumin, 1×10^{-5} *M*. $CuCl_2$, 5×10^{-4} *M*. Buffer in the dialysis equilibration, acetate, pH 4.7, 0.1 *M*; pH 5.7, 0.16 *M*; pH 6.5, 0.2 *M*

X-ray dose, r.	Number of Cu^+ ionins combining with albumin		
	pH 4.7	pH 5.7	pH 6.5
0	3.1	5.5	8.2
10,000	3.2	6.3	8.8
20,000	3.4	6.8	9.6
40,000	3.5	7.8	10.8
60,000	3.4	8.8	11.7

by Longsworth.[22] At pH 8.9, the sharp and symmetrical boundaries of both ascending and descending limbs showed, after X irradiation, lack of enantiography, the ascending limb becoming somewhat broader. X irradiation of globulin caused a slight decrease of electrophoretic mobilities, except at pH 8.9, where there was an increase. The shape of the boundaries was not changed at pH 3. At other pH values, however, although the boundaries remained symmetrical, they became more diffuse in both ascending and descending limbs (TABLE 7).

Serum albumin contains one —SH group per molecule.[23] It may be possible to form a dimer, protein S—S protein, on oxidation of the —SH groups by X irradiation. In sedimentation in the analytical Spinco ultracentrifuge of serum albumin irradiated with 100,000 r., a second component appeared with a con-



Irradiation Of Limulus Hemocyanin. Conc. 1.96%

Abscissa, wave length in mμ

Ordinate, optical density.

1. Unirradiated hemocyanin.
2. Hemocyanin irrad. with 400,000 r.
3. Hemocyanin irrad. with 600,000 r.

FIGURE 8

TABLE 7

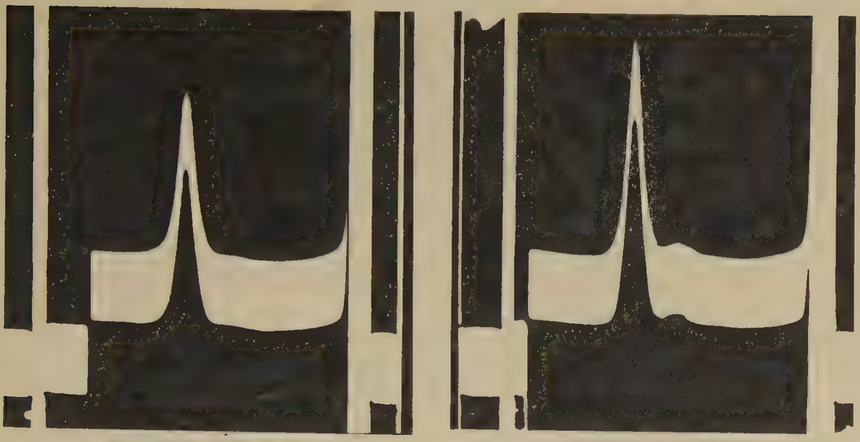
EFFECT OF X IRRADIATION ON THE ELECTROPHORETIC MOBILITY OF PROTEINS

The protein, 1 per cent, was dissolved in 0.1 *M* phosphate buffers, irradiated at 10° and the mobilities measured at 0°. X-ray dose, 100,000 r. except those marked with an asterisk, where it was 200,000 r.

Protein	pH	Mobility ($\mu \times 10^5$)		Sp. conductivity ($k \times 10^3$)	
		Control	X ray	Control	X ray
Albumin (dialyzed)	3.0	8.56	8.30	5.75	5.68
Albumin (not dialyzed)	3.0	8.90	8.15	5.68	5.94
	3.0	8.90	6.75	5.68	7.80
	4.47	0	0	5.60	5.52
	7.0	4.68	4.81	6.88	8.45
	7.0	4.68	4.24	6.88	10.32
	8.9	5.62	5.23	8.14	8.91
Globulin (not dialyzed)	3.0	6.38	5.83	5.56	6.12
	6.0	0	0	6.80	6.17
	7.0	1.53	1.50	7.15	7.97
	7.0	1.53	1.25	7.15	6.96
	8.9	1.56	1.95	8.09	8.95

centration of 20 per cent, which seems to be a dimer (FIGURE 9). Addition of cysteine (0.003 *M*) prevented the appearance of the second component.

A number of investigators have irradiated amino acid solutions with large doses of X rays,[24, 25] alpha rays,[26] and cathode rays.[27] Dale[24, 27] measured the formation of NH_3, H_2, and aldehyde. From such experiments, Stein and Weiss attempted to find the initial reaction products. These experiments were repeated with lower concentrations of amino acids (1×10^{-4} *M*) and lower radiation intensity (48,000 r.). Histidine and tryptophan were deaminated



a b

FIGURE 9

TABLE 8

DEAMINATION OF DILUTE AQUEOUS SOLUTIONS OF AMINO ACIDS BY X IRRADIATION

The Effect of Oxygen

Amino acid concentration, 1×10^{-4} *M*. X-ray dose, 46,000 r.

Amino acid	NH₂ lost, μM × ml.			
	In O₂	G	In N₂	G
Histidine-HCL	0.051	0.415	0.03	0.24
Tryptophan	0.05	0.41	0.025	0.2
Tyrosine	0.027	0.217	0.012	0.097
Phenylalanine	0.036	0.29	0.022	0.177
Serine	0.039	0.315	0.028	0.23
Threonine	0.032	0.26	0.022	0.18
Lysine-HCL	0.033	0.265	0.025	0.204
Arginine	0.038	0.314	0.029	0.237
-Alanine	0.01	0.08	0.0045	0.035
-Alanine	0.008	0.064	0.005	0.043
Glycine	0		0	

to the extent of 50 per cent; then came serine and arginine, 38 per cent; phenylalanine, lysine, threonine, and tyrosine, around 30 per cent; and α and β-alanine, around 10 per cent. In every case, irradiation of oxygen-saturated solutions of amino acids was more effective than irradiation of nitrogen-saturated solutions (TABLE 8).

X irradiation of glycine produced no deamination. When the concentration was increased (0.1 *M*), irradiation of oxygen-saturated solutions of glycine gave, at pH 9, equal amounts of glyoxylic acid and of NH_3 (TABLE 9). From these experiments, it is plausible to postulate that the *initial* reaction on X irradiation of amino acids is oxidative deamination, performed by the two oxidizing radicals, OH and O_2H, the first producing oxidation to the half-oxidized

TABLE 9

X IRRADIATION OF CONCENTRATED SOLUTIONS OF GLYCINE

X-ray dose, 50,000 r.

The lower and higher pH values were obtained by addition of HCL or NaOH. The figures give μM per ml. after irradiation

pH	NH_3	Glyoxylic acid
5.8 (water)	0.05	0.11
3.0	0.11	0.095
9.0	0.09	0.08

amino acid, the second completing the oxidation to the imino acid:

$$CH_2NH_2COOH + OH \rightarrow \dot{C}HNH_2COOH + H_2O$$

$$\dot{C}HNH_2COOH + O_2H \rightarrow CH{=}NHCOOH + H_2O_2$$

The H_2O_2 produced in the second reaction would be ready to oxidize more amino acid if traces of Fe^{++} or Cu^{+} ions were present in the solution to provide the reactive OH radicals by means of the Fenton reaction.[28, 29] The amino acid would spontaneously hydrolyze in the presence of water to give glyoxylic acid and ammonia:

$$HC{=}NHCOOH + H_2O \rightarrow HCOCOOH + NH_3$$

The formation of formaldehyde, formic acid, H_2, and CO_2 on X irradiation of glycine with large X-ray doses can no longer be called a product of the initial reaction, because these substances are produced by the further oxidation or decarboxylation of glyoxylic acid and the further oxidation of formic acid (produced on oxidation of glyoxylic acid). These substances may even be a product of enhancement, as previously described.

Nucleic acids absorb light strongly at about 2,600 Å. because of the purine, pyrimidine, and adenine residues contained in the molecule. X irradiation of these components as well as of nucleic acids produced a decrease in the absorption spectrum before other changes were observed.[19] Irradiation of cytosine, for example, with 50,000 r. greatly reduced the absorption spectrum. Addition of pentose to the nucleoside to form cytidine exerted a marked protective action. Similar protection was observed on irradiation of adenine, its nucleotide adenosine, and the phosphorylated derivatives adenylic acid, adenosine diphosphate, and adenosine triphosphate. As the complexity of the molecule increases, *i.e.*, as more and more groups are added to the purine or pyrimidine nucleus, the X-ray effect on the absorption spectrum diminishes (TABLE 10). The radiochemical yield of DNA, for example, was 1 to 4000 times less than the radiochemical yield of the purines contained in the DNA molecule. This relative protection is perhaps due to the structure of the molecule, which, according to recent investigations,[30-32] seems to be helical, the phosphate groups lying on a helix of a diameter of about 20 Å., and the sugar and base groups turned inward toward the helical axis. I believe this finding is an indication that the cellular nucleoproteins are more resistant to irradiation than heretofore postulated.

TABLE 10

RADIOCHEMICAL YIELD ON X IRRADIATION OF PURINES AND PYRIMIDINES IRRADIATED IN DILUTED AQUEOUS SOLUTIONS

Substance	G (100 e.v.)
Sodium deoxyribonucleate	0.00385
Sodium ribonucleate	0.00724
Thymine	1.245
Uracil	0.64
Guanine	0.261
Cytidine	0.616
Cytosine	1.845
Adenine	0.676
Adenosine	0.196
Adenylic acid	0.161
Adenosine diphosphoric acid	0.138
Adenosine triphosphoric acid	0.109
Uric acid	0.37
DPNH (oxidation)	1.51
DPN	0.02

X Irradiation and Cellular Respiration

A study of the rates of reaction of isolated, oxidation-reduction enzymes and the rates of cellular respiration has shown that the former are always much higher than the latter. This difference is due to the presence in the cell of regulatory mechanisms, which increase in number and in complexity the higher we go in the phylogenetic scale. Of these regulatory mechanisms, the nonprotein —SH groups, such as glutathione, are the most universally distributed. Small amounts of —SH reagents, enough to destroy glutathione, will then cause an increase in respiration, whereas large amounts will produce inhibition of respiration by combining with the —SH enzymes necessary for a large number of oxidations. This difference was found in a study of the effect of —SH reagents on the respiration of sea urchin sperm.[33] If X irradiation attacks preferentially —SH groups in the cell, it could produce the same effects as those produced by the —SH reagents. X irradiation of fertilized sea urchin eggs with 1,000 r. inhibited respiration. When X-ray dose was diminished to 200 and 100 r. there was an increase (TABLE 11). Similar results were obtained on X

TABLE 11

EFFECT OF X IRRADIATION ON THE RESPIRATION OF FERTILIZED SEA URCHIN SPERM

Temp. 28°; time, 2 hours

X-ray dose	O_2 Uptake		Inhibition (−) or increase (+)
	Control	Irradiated	
	c.mm.	*c. mm.*	*per cent*
1,000	26.8	16.9	−37
500	34.4	26.6	−22.5
200	51.6	55.4	+7.4
100	42	50.0	+19

irradiation of sea urchin sperm.[34] On fertilization, the rate of cleavage of the fertilized eggs was inhibited even on irradiation with X-ray doses as small as 100 r. These experiments demonstrate the importance of the —SH groups in the interpretation of the biological effects produced by small X-ray doses. The inhibition of cellular growth and multiplication produced by ionizing radiations must be due to inhibition of very labile —SH enzymes necessary for the synthesis of nucleic acids.

L. H. Gray, in his excellent address to the Radiation Research Society,[35] stated that "since the total number of oxidizable —SH groups in the cell has been estimated by Patt to be of the same order as the number of molecules decomposed by a million roentgens, we cannot expect to find, *a priori*, that a dose of a few hundred roentgens will significantly reduce the activity of any one type of —SH-containing molecules." Unfortunately, Gray did not take into consideration that —SH-containing molecules behave differently towards oxidizing agents according to the distance between the groups, the presence of electronegative groups, and other steric hindrances.[36] X irradiation does *not oxidize* all —SH groups. Ionizing radiations oxidize *only* those freely reacting —SH groups, leaving the others untouched. When we irradiated mixtures of alcohol dehydrogenase, D-amino acid dehydrogenase, and hexokinase (all —SH enzymes) with 100 r., the first enzyme was 90 per cent inhibited, whereas the other two remained unaffected. Titration of total —SH groups is, therefore, no indication of the action of ionizing radiations when the freely reacting groups are in small amounts compared to the total —SH groups.

I should like finally to emphasize that ionizing radiations of large intensity produce effects *qualitatively different* from radiations of intensities around the lethal dose, and that attempts which have been made lately to extrapolate the effects produced by large doses of ionizing radiations to effects that may be produced by small doses are erroneous.

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Discussion of the Paper

Doctor K. Salomon (*The University of Rochester, Rochester, N. Y.*): First: concerning the experiments reported in this paper on hemocyanin, I wonder whether attempts were made to reconstitute the complete hemocyanin molecule after the experiment.

Second: I was especially interested in the absorption spectra of hemoproteins shown in this paper, since they show clearly that the heme as well as the protein moiety of these substances are affected by ionizing radiation. It might be of interest in this context to mention experiments which have been carried out in our own laboratory on the biosynthesis of hemoglobin in intact animals. About 800 r. were applied to rabbits, and their bone marrow and spleens were taken out at varying time intervals after radiation. Bone marrow and spleen homogenates were incubated in the presence of α-carbon C^{14} glycine, and hemoglobin was isolated from these mixtures. It could be shown, after isolation of hemin and globin, that both hemin and globin synthesis are affected by ionizing radiation, but to a different degree. In contradistinction to the effects shown by radiation on hemoproteins in solution, our findings are probably due, in the last analysis, to the effect of the radiation on the enzyme systems concerned with hemin and globin synthesis *in vivo.*

Doctor Barron: I think Doctor Salomon's suggestion of trying to reconstitute the hemocyanin molecule is a good one. We shall attempt to do it.

Doctor G. Weber (*Institut du Cancer de Montreal, Montreal, P. Q., Canada*): I should like to ask you a question regarding your statement on the sensitizing effect of lactic acid, which you observed in *in vitro* experiments. As it is known that neoplastic tissue is rich in lactic acid, could it be possible that this increased lactic acid content might play a role in the increased radiosensitivity of the cancerous tissue?

Doctor Barron: It is quite possible that oxidation of lactic acid in the tissues by ionizing radiation may produce the enhancement observed *in vitro.*

Doctor M. A. Lessler (*Ohio State University, Columbus, Ohio*): You mentioned that *Arbacia* egg and sperm show decreased respiration following radiation dosages above 100 r. and increased respiration following doses less than 100 r. Do you consider that 100 r. represents a type of radiation inflection point for living material? Second, although there is evidence that simple isolated biochemical compounds are damaged by lower dosages of ionizing radiation than more complex compounds, there is a great deal of evidence which indicates that complex mixtures of compounds (such as occur in a living cell) are damaged by much lower levels of radiation than the isolated compounds.

Doctor Barron: Although the increase of respiration on X irradiation of fertilized sea urchin eggs and sperm came on irradiation with 100 r., on unfertilized sea urchin eggs it happened with 500 r. The limiting dose must change from cell to cell. With reference to your second question, it is still too early to understand what are the systems initially attacked by ionizing radiations. More work of the kind I have presented here remains to be done.

Doctor W. R. Guild (*Yale University, New Haven, Conn.*): The point was made that the larger the molecule, the greater its apparent resistance to radiation damage, as measured by a change in its absorption spectrum. An example mentioned was DNA as opposed to nucleotides, nucleosides, and the purine and pyrimidine bases.

Do you not think that this behavior merely reflects the decreasing probability of the chemical effect occurring in a group which is spectroscopically important? It would seem likely that very little could be done to a purine without changing its spectrum, whereas, as the size of the molecule increased, there would be many places for bonds to break without affecting the visible or ultraviolet spectrum.

The most sensitive indicator of radiation damage is biological activity. As Setlow has pointed out in a previous paper, the direct-action ionic yield is about unity over a range from penicillin (M.W. 350) to pneumococcus transforming principle DNA, of molecular weight about 6 million. This means that one ion pair anywhere in the molecule inactivates its biological function, regardless of size of the molecule. Since, according to Barron's data, the spectrum does not change markedly, the implication seems to be that the significant damage does not have to affect a spectroscopic group.

Doctor Barron: I should like to give two more examples in favor of my contention of the protection of sensitive groups, and of the relative action of ionizing radiations: Fe^{++} ions in $FeSO_4$ are oxidized with a radiochemical yield G of 15; in cytochrome *c* (M.W. 13,000) with a G value of 1.6; and in hemoglobin (M.W. 68,000) with a G value of 0.08. The —SH groups of cysteine are oxidized with a G value of 10, the —SH group being largely oxidized without the NH_2 group being attacked. Deamination occurs only on irradiation with large X ray doses, and the G value is only 0.9, *i.e.*, 10 times less than oxidation of the —SH group.

STUDIES ON FACTORS AFFECTING THE SENSITIVITY OF BACTERIA TO HIGH VELOCITY ELECTRONS

By W. D. Bellamy and E. J. Lawton
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The development of reliable sources of intense high velocity electrons[1, 2, 3] as well as the production of large amounts of radioactive waste[4] have caused renewed interest in sterilization by ionizing radiation. Knowledge of factors affecting the sensitivity of microorganisms to radiation becomes of practical importance when attempting to sterilize food, drugs, and related products with minimum radiation doses. It is hoped, however, the basic information obtained from the study of irradiated bacteria will have application far beyond the immediate problem of radiation sterilization.

This report covers some of the experiments carried out over the past several years at the General Electric Research Laboratory with HVe, with the cooperation of Mary Germain and John Balwit.[5] The source of HVe is a 1 MeV resonant transformer X-ray unit converted to an electron source by replacement of the heavy tungsten target with a thin (2 mil) stainless steel window.[1] Operating at 800 kv. (peak) and 100 microamperes beam current, a dose is accumulated at the rate of 143,000 rep per second in a target 10 cm. from the window. The beam is completely stopped in 3 mm. of water. A 2-gram sample spread over a 2-inch circle (0.1 gram per $cm.^2$) 10 cm. from the window receives a dose which does not vary by more than 15 per cent from center to edge and from top to bottom.

It has been necessary to adapt bacteriological methods to irradiate samples in aqueous layers of 1 mm. or less, and it has been found feasible to absorb and/or dry bacterial suspensions on filter paper for irradiation. The results of a few of these experiments are presented below. We have found no essential difference in the survival of *Escherichia coli* B/r to X rays at 1000 rep per minute and to HVe at 143,000 rep per second. We have interpreted this independence of dose rate over an 8000-fold range to mean that, for this organism at least, a chain reaction initiated by free radicals is not involved. It has been shown that reactions which are both initiated and terminated by free radical mechanisms are rate sensitive. An example of this is the polymerization of tetraethylene glycol dimethacrylate (TEGMA).[6] It was found that the physical quality of the polymer is profoundly influenced by the rate of dose accumulation. For example, a dose of 0.2×10^6 rep accumulated in 10 seconds produced a liquid somewhat more viscous than the monomer. Decreasing the dose rate resulted in a product which passed from a weak gel through a thick gel to a solid. The same dose (0.2×10^6 rep) accumulated in 200 seconds produced a hard solid. If the dose rate is high, the radicals terminate the chain before large polymers can be formed while a low dose rate favors longer chains. The only reaction of biological importance that has been reported to be rate dependent is the oxidation of unsaturated fats.[7] Apparently this reaction is not important in the survival of *E. coli*.

Irradiation of *Staphylococcus (Micrococcus) aureus* 209 in liquid suspension, frozen and dry, produce first-order survival curves. However, the mean lethal dose increased from 5000 to 6000 rep for liquid cell suspensions to about 25,000 rep for cells irradiated at −78° C. The target diameter calculated after the manner of Lea[9] was found to be 95 to 105 millimicrons for aqueous suspensions of *S. aureus* 209. The sensitive volume is therefore about one one-thousandth of the entire cell volume. The target diameter of the frozen organism is about 0.6 that of the bacteria in aqueous suspension. The MLD for dry *S. aureus* irradiated at room temperature is slightly higher than for frozen cells at −78° C. Spores of *Bacillus subtilis* were found to be more resistant to radiation than vegetative cells but were only slightly more resistant than dry or frozen staphylococci. In contrast to the vegetative cells, the *B. subtilis* spores were found to be only slightly affected by freezing or drying. *Bacillus larvae*, the cause of foul brood in bee hives, forms one of the most heat resistant bacterial spores known. The next slide indicates that it is also quite resistant to ionizing radiation. It is apparent that the spores consist of at least two species of radiation resistance, about 10 per cent of the spores are many times as resistant as the majority[8] (FIGURE 1).

Most of the radiation studies on bacteria have been done with organisms such as *E. coli* because of the ease of culturing these bacteria and because they do not grow in clumps or chains. Lea[9] warned against the use of organisms such as *Staphylococci* because the survival curve will not be exponential but will be sigmoid. If the proportion of organisms which survive a given dose is the exponential function e^{-x} where x is proportional to the dose, then the probability that an individual organism shall be killed by thise dose is $1 - e^{-x}$. If the organisms are in clumps or chains of n individuals, the probability that all n organisms of a clump are killed is $(1 - e^{-x})^n$, and the proportion of clumps producing colonies after a dose proportional to x will be $1 - (1 - e^{-x})^n$. The latter function is not exponential but sigmoid.

There are several factors which make certain of the streptococci most useful for a study of the mechanism of radiation damage. Among these factors is the complete lack of an aerobic respiratory enzyme system and the lack of storage products, such as starch and fats. These streptococci have no enzyme system, such as catalase, for the destruction of hydrogen peroxide, although many can tolerate relatively high concentrations of peroxide. In fact, many of them, in the presence of air, produce inhibitory amounts of hydrogen peroxide through the so-called flavine leak.

It seemed worth while to determine if these microorganisms, lacking a complete aerobic respiratory system, would respond to oxygen during irradiation, as does an organism having a cytochrome system.

Preliminary examination made by the Knaysi staining[10] technique for living and dead cells indicated that the average number of living bacteria per chain was two for both *Streptococcus faecalis* 10Cl and for *Streptococcus lactis* L21S, when grown 18 hours in a medium of 1 per cent yeast extract, 1 per cent hydrolyzed casein, 0.5 per cent potassium phosphate, and 0.1 per cent glucose. According to typical survival curves of these organisms, as shown in FIGURE 2, *S. lactis* is in the same order of sensitivity as *E. coli* B/r, but *S. faecalis*, an organism which is more resistant to heat, to dyes, and to salt concentrations, is

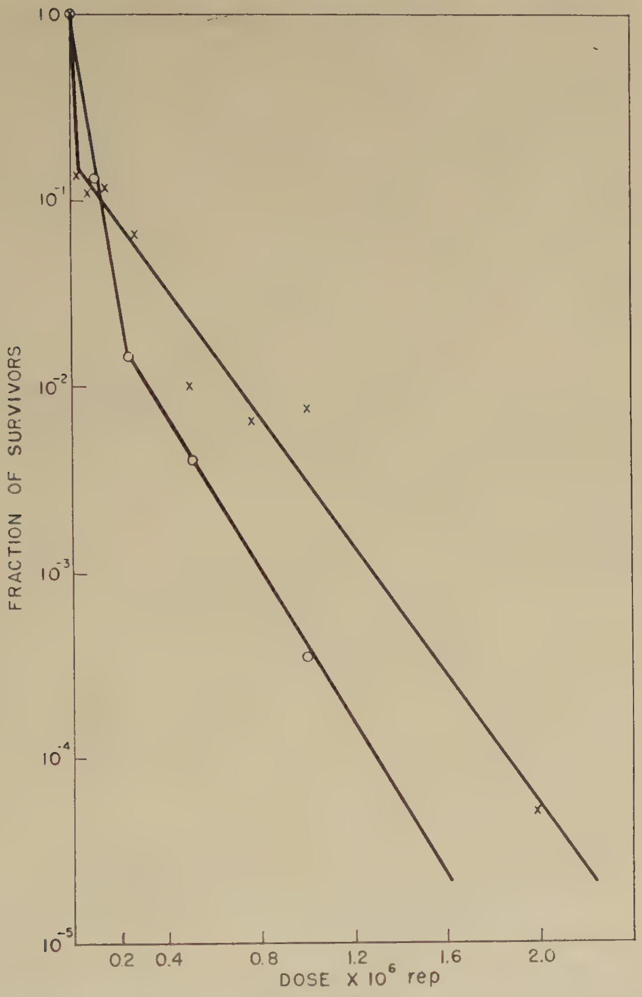


FIGURE 1. Plot of the logarithm of survivors of *Bacillus larvae* spores versus dose of HVe. The dose was accumulated at the rate of 140,000 rep per second. Irradiated in honey at room temperature. Original number of spores was (X) 3×10^6 per ml. and (O) 4.2×10^6 per ml. of honey.

much more resistant to radiation. The latter microorganism, in fact, is about ten times as resistant as B/r or L21S. It can be seen that the curves will extrapolate back to an *n* number of 2 to 5, which is in reasonable agreement with the direct count of living cells per chain. *S. faecalis* 10Cl, when dried in mass from suspensions in phosphate buffer, exhibits the expected increase in resistance over liquid suspensions, although the difference is not as great as with *E. coli* or *Staph. aureus* 209.

If *S. faecalis* cells are grown on the above medium and washed with phosphate buffer, they have very little storage product, as indicated by their inability to to reduce methylene blue when tested by the Thunberg technique. Such cells are as sensitive to irradiation in air as are *E. coli* cells. If the cells are not

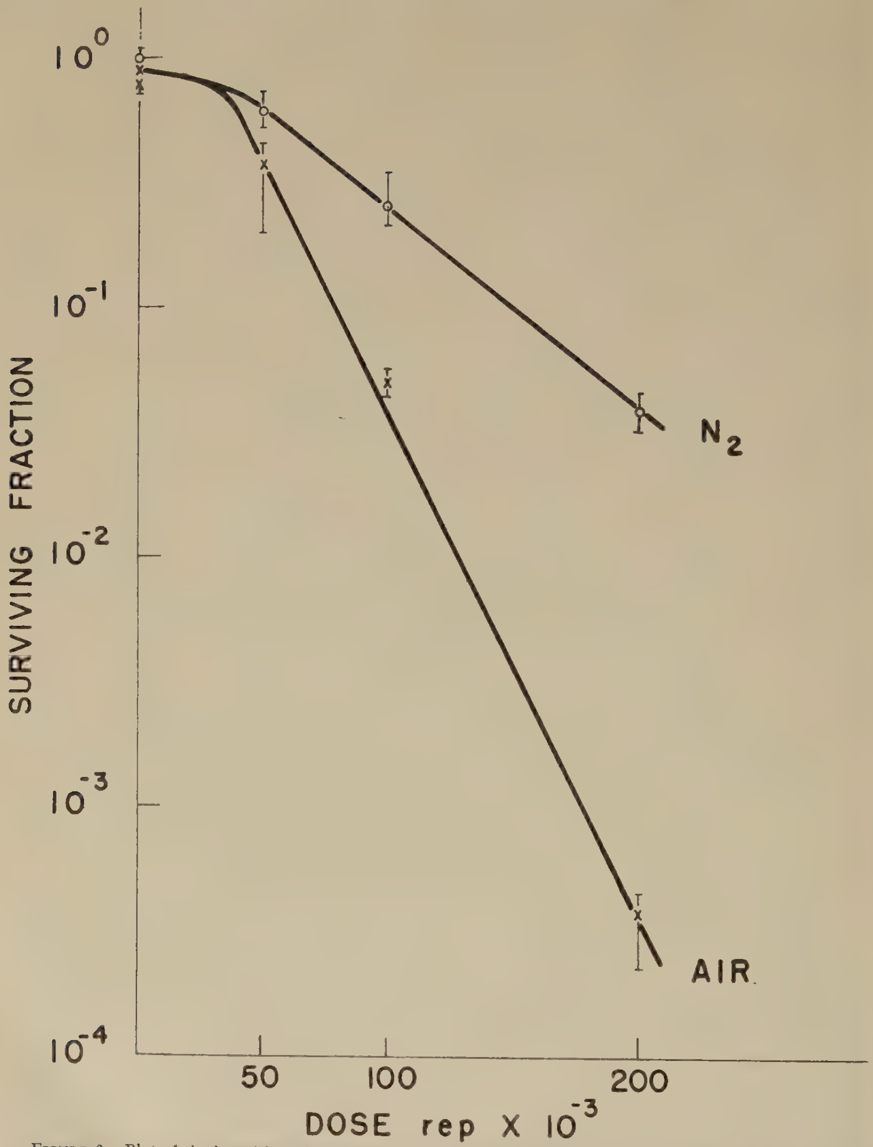


FIGURE 2. Plot of the logarithm of survivors of *S. faecalis* 10Cl versus dose of HVe. Cells grown 18 hours at 25° C., irradiated in phosphate buffer at room temperature in stainless steel dishes. Dose accumulated at the rate of 140,000 rep per second.

thoroughly washed, or if glucose is added previous to irradiation, there is decreased sensitivity to air. If glucose and methylene blue are added previous to irradiation, however, there is no protection from air. It is well known that MB mediates the transport of hydrogen to oxygen, resulting in the production of H_2O_2.

Membrane filters have been found very useful for the study of radiation of very limited penetration. The membrane used in these studies is manufactured by the Lovell Chemical Company of Watertown, Mass., and marketed under the name of Milliport filters. The Milliport filter (MPF) is made of nonfibrous material having a maximum pore size of $<0.2\ \mu$ and is 80 per cent voids. It is possible to culture the bacteria on the filters, after irradiation, by

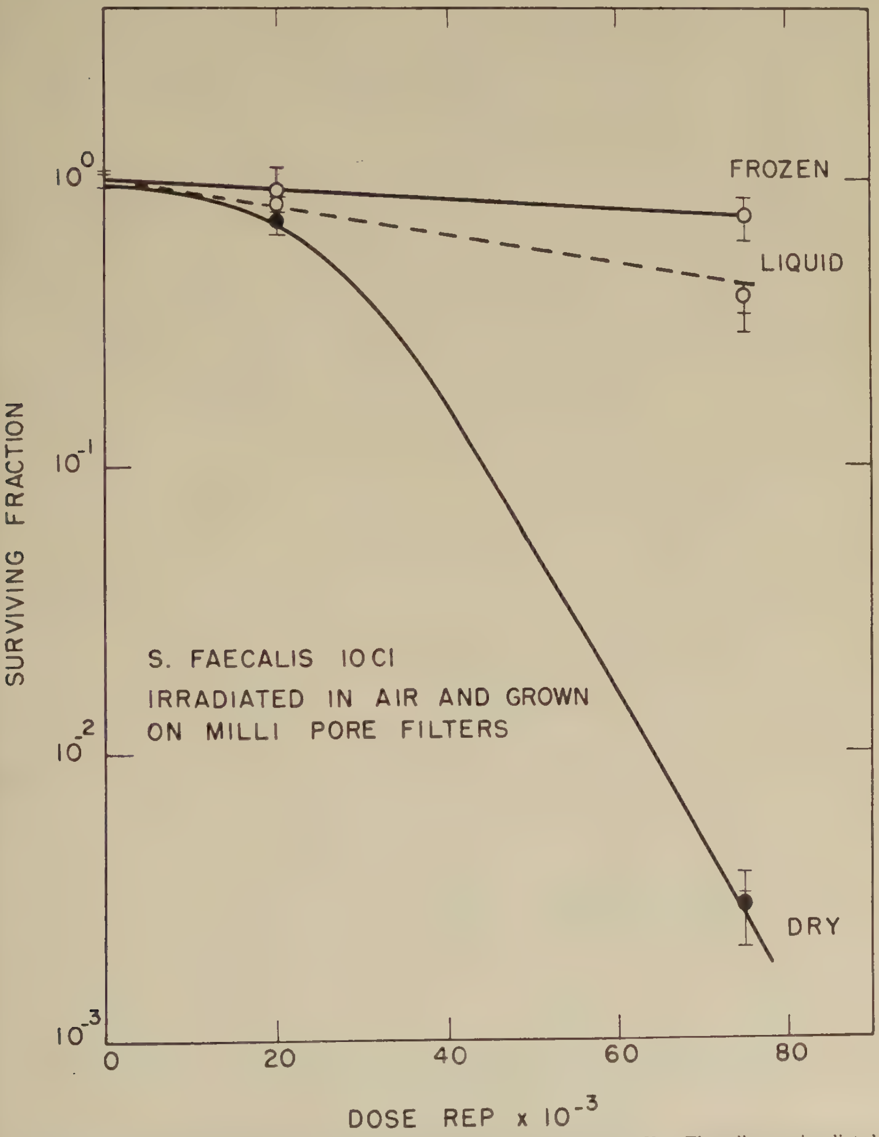


FIGURE 3. Plot of logarithm of survivors of *S. faecalis* 10Cl versus dose of HVe. The cells were irradiated on millipore filters in air (a) in frozen broth at −78° C., (b) in liquid broth at 25° C., (c) dry at 25° C.

placing the filter on a nutrient pad, thus eliminating the usual diluting and plating techniques. The bacterial cells can be pictured as growing at the top of a column of nutrient broth obtained from the reservoir below. This environment is entirely different from a broth culture, and both growth response and irradiation sensitivity are quite different. The filter can be readily moved from one culture medium or condition to another. It is thus possible to subject the bacteria to a variety of conditions before and after irradiation.

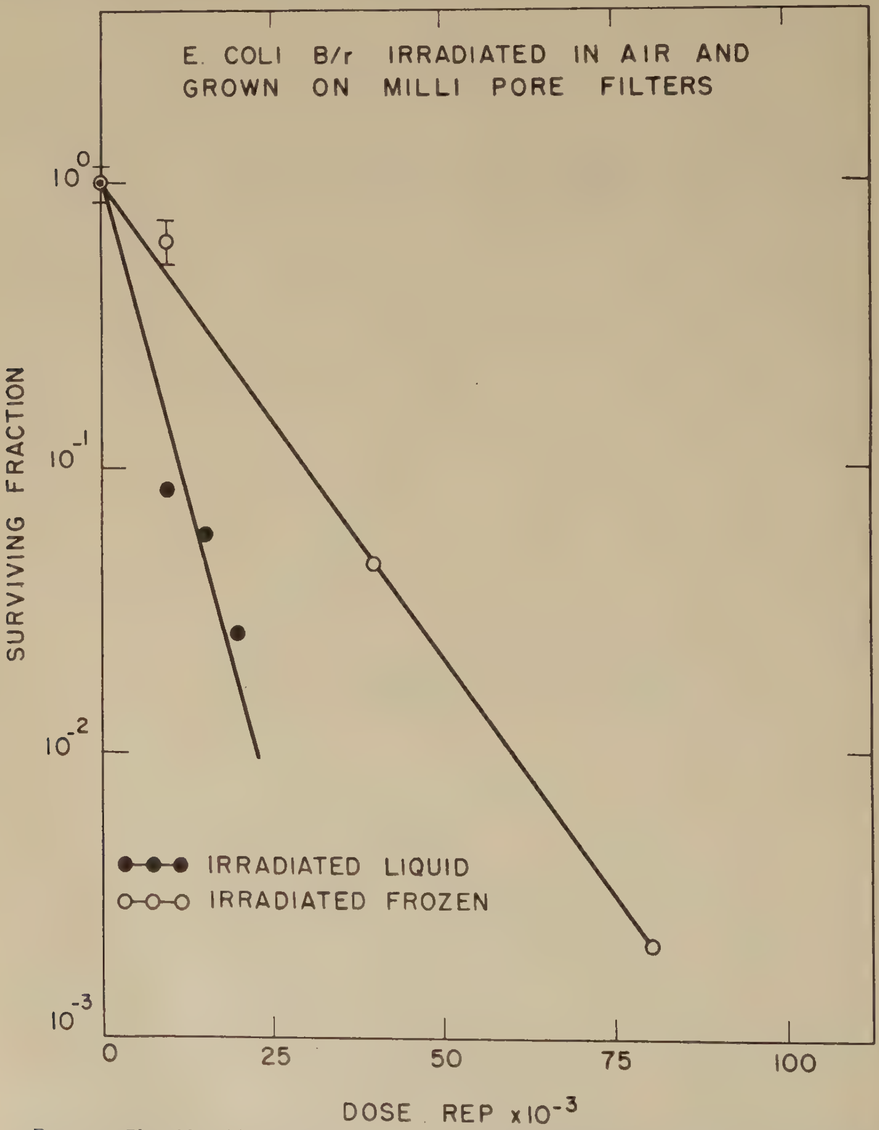


FIGURE 4. Plot of logarithm of survivors of E. Coli B/r versus dose of HVe. The cells were irradiated on millipore filters in air (a) in frozen broth at −78° C., (b) in liquid broth at 25° C.

S. faecalis will survive freeze drying *in vacuo* on the filters. FIGURE 3 shows a comparison of the survival of *S. faecalis* on MPF's irradiated in liquid broth, frozen in phosphate buffer, and dried from phosphate buffer. The increased sensitivity of dried cells was unexpected, but has been found on incubation of 10°, 34°, 45° C.

Perhaps the excitation energy travels farther through the molecular structure of a dry streptococcus than it does through a completely hydrated organism (*cf.* Pollard and Setlow elsewhere in this monograph). The molecules of the solvent may absorb much of the vibrational energy. If this is the case, it does not explain why the same organism, when dried in mass rather than on MPF's, is more resistant dry than hydrated.

E. coli B/r will not survive freeze drying under the above conditions, but will survive freezing. FIGURE 4 shows that *E. coli* on MPF's is much more sensitive when irradiated in liquid than when frozen in broth or in phosphate.

S. faecalis 10Cl, dried on MPF's, were not measurably more sensitive to irradiation in air than to irradiation in nitrogen. This finding confirms the theory that oxygen acts indirectly through irradiated water.

The effect, on removal of the water by immobilization, is shown in FIGURE 5. A dilute solution of pepsin was irradiated with a 2.5 × 10^6 rep at temperatures from −190° to 30° C. It is apparent that the inactivation is 90 per cent when the solvent is liquid, and 10 per cent when solid.

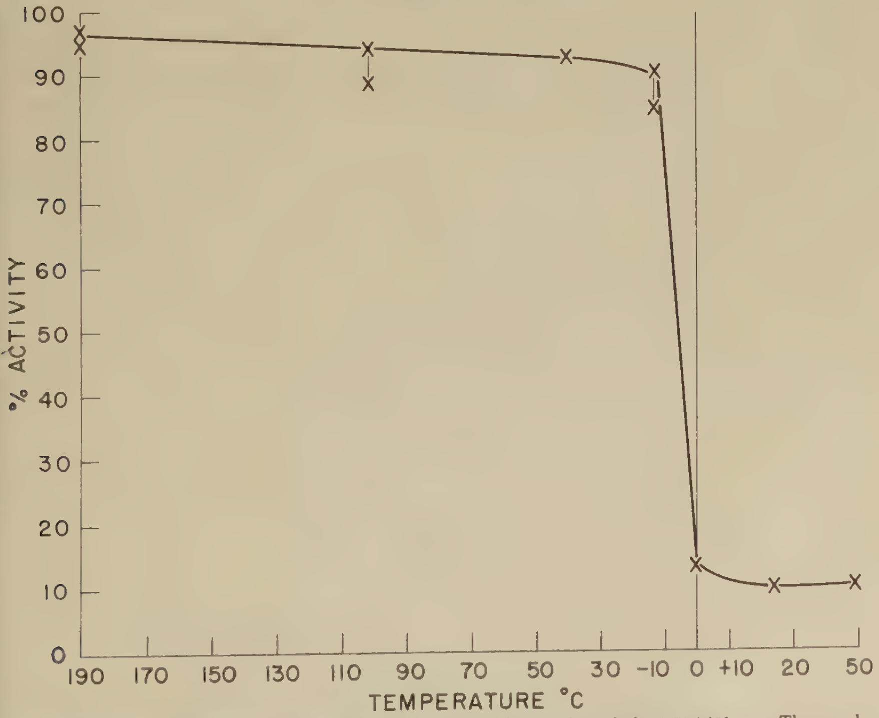


FIGURE 5. Effect of temperature on the inactivation of a solution of pepsin by 2.5 × 10^6 rep. The samples were irradiated in air in stainless steel dishes. Temperatures were determined by a recording thermocouple. The pepsin was irradiated at 10 mgm./ml. in water and assyed for activity at 0.5 mgm./ml.

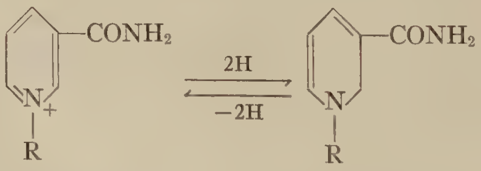
In the above example, about 90 per cent of the inactivation is contributed by the active radicals produced in the solvent when the solvent molecules are free to migrate. Whether this is the case in living cells is not known. However, a very simple experiment has been done which seems to indicate that much of the effect in living cells must be direct. Crushed apples were irradiated in air to determine the dose necessary to prevent browning by the action of tyrosinose or polyphenol oxidose. At room temperature, the inactivation dose is 5 to 7 × 10^6 rep. If the apple is irradiated at −78° C. the inactivation dose increases to about 10 × 10^6 rep. These results indicate the great amount of protection provided by the many cell constituents.

Discussion

Any theory explaining the radiation effects on microorganisms must combine these isolated observations as well as those in the literature into a unified, consistent whole. The theory will have to explain the observations of Alper[12] and of Bachofer and Pottinger[13] that under certain conditions bacteriophage appear to be protected by oxygen. The studies of Rubin[14] at Brookhaven showed that growing *E. coli* is unaffected by continuous radiation as high as 6000 rep per hour. Beam et al.[15] have recently reported that yeast cells increase in resistance manifold during the process of budding. Starving of the yeast cells was shown to decrease their resistance to radiation. The authors interpreted their survival curves to indicate that the number of sensitive sites and the degree of replication had increased about 1000-fold during the process of budding. An alternative interpretation is that the dividing yeast cell produces many working molecules which must be destroyed before a "sensitive site" can be inactivated.

We suggest that the following mechanism is superimposed on the well-established genetic effect. The protection offered by the addition of glucose or other metabolite to a culture of *E. coli* or *S. faecalis* is not due solely to the removal of molecular oxygen but is due to the reduction of the enzyme systems and some other constituents of the cells. It has been well established that the respiratory enzymes function by alternate oxidation and reduction, and this process has been shown to take place by single electron transfer with free radical intermediates. For example see Michaelis.[16]

Coenzyme 1 can be used as an example of what must occur on a larger scale within the cell



Sensitive to
oxidizing agents

More sensitive when
irradiated in presence
of oxygen.

Sensitive to
reducing agents

No increased sensitivity
when irradiated in presence
of oxygen.

Thus any condition which will drive the reaction to the left will increase the oxygen sensitivity. Among these we can mention increased concentation of oxygen, starving the cells, *i.e.* depleting the source of available reducing [H], and adding oxidizing agents.

Anaerobic organisms have been shown to grow at a much lower oxidation reduction potential than aerobes. Under such conditions a large fraction of the cell components are reduced and therefore less sensitive to oxygen when irradiated. It is characteristic of biological oxidation-reduction systems that they react only with the system immediately above or below and are not autoxidizable. Therefore, they will remain either reduced or oxidized in the absence of substrates.

Conclusion

There is a great species difference in radiation sensitivity. In general, those organisms more resistant to heat are also more resistant to irradiation. The growth conditions greatly affect the sensitivity of most microorganisms. One of the many factors involved is the oxidation-reduction potential of the cells. Reduced systems are much less sensitive to radiation in the presence of oxygen than are oxidized systems. Evidence is presented showing the difficulty in extrapolating from radiation effects on an isolated enzyme system to an intact cell.

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THE INFLUENCE OF PRETREATMENTS AND POSTTREATMENTS ON BACTERIAL INACTIVATION BY IONIZING RADIATIONS

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An evaluation of the available information concerned with the mode of lethal action of ionizing radiations on bacterial cells leads to the conclusion that alteration of relatively few molecules within the cell must ultimately bring about its inactivation. It is possible that a chain reaction is initiated by the radiation that results in the destruction of a large number of biologically important molecules. The previous paper has discussed the improbability that a radiochemical chain reaction is of importance in the inactivation of bacterial cells. It has been proposed in the past that the effect we study, usually the destruction of the ability of a bacterium to form a visible colony, may be the consequence of a biochemical chain reaction, in that some key synthetic system may be disrupted by the radiation, which leads to a general loss of synthetic ability of the cell. The final answer to what happens between the initial physical events and the biological effect that we study may be forthcoming by an orderly bringing together of the results of the research based on the diversified approaches now being used.

The general conclusion from the radiobiological experimentation involving the use of bacterial cells as test organisms is that the inactivation results chiefly, if not entirely, from energy absorption within the cell. This conclusion is based primarily on the general finding that the fraction rather than the number of cells inactivated per unit dose is constant, independent of the concentration of bacteria in the irradiated suspension, and also independent of added protein in the suspension. Both lines of evidence indicate that the absorption of energy in the surrounding medium contributes very little to the lethal effect.

It is well to indicate, at this point, that a variety of agents which may be added to a suspension of bacteria before and during irradiation can alter the sensitivity of the bacteria to ionizing radiation.[6] Changes in the conditions surrounding the bacteria during irradiation can also modify the bactericidal effect. These treatments may be divided into at least two classes, depending on when they may be effectively used with respect to the irradiation period. This division is somewhat arbitrary, since many techniques for measuring the damaging effect (for example, viable cell count) do not usually permit a decision as to whether the modifying system had its effect before, during, or after the radiation exposure.

Most of the data to be presented in this paper will be concerned with the lethal action of ionizing radiations on bacterial suspensions. In most cases, this action has been measured by the radiation-induced loss of ability of the cells to reproduce or form colonies on a growth medium. In all cases the bacteria, unless otherwise specified, are stationary-phase *Escherichia coli*, usually from a continuously aerated nutrient broth culture about 18 hours old. The irradiation was usually carried out with 250-kvp. X rays at a dose rate of about 2000 r/minute.

Anderson and Turkowitz[1] recognized that inactivation of yeast by gamma rays was dependent on the oxygen content of the irradiated suspension. These investigators found that there was a remarkable difference in sensitivity between yeast grown in stationary culture and those cultured with aeration, the aerated culture being the more sensitive, and attributed the difference to the amount of oxygen in the cultures. The generality of this phenomenon has now been clearly demonstrated.[6] It is clear from the published data that about three times the amount of radiation is required to bring about equivalent lethal effect in the absence of oxygen as in the presence of this gas. That the effect of reduction of radiation sensitivity is related to oxygen removal and not to the gas used to displace the oxygen is indicated by the finding that flushing the suspension with any inert gas produces the same reduction in sensitivity. A variety of chemical compounds which have the ability to react with dissolved oxygen bring about a similar quantitative reduction in lethal effect per unit dose. Notable among these compounds are sodium hydrosulfite ($Na_2S_2O_4$) and some sulfhydryl compounds, among the most efficient of which is BAL (dimercaptopropanol).[6] It has been shown that the same quantitative effect can be brought about by allowing the cells to use up their own contained oxygen when incubated at 37° C. in the presence of an oxidizable substrate before irradiation.[11] The effect seems to be chiefly one of oxygen removal, since the protection effected by this system can be eliminated if respiratory inhibitors are included in the incubated mixture prior to irradiation. The protection afforded by this system could be eliminated also by flushing the system with oxygen just prior to irradiation. Either drying or freezing of the suspension prior to irradiation has been shown by Moos,[8] Wood,[16] and by Stapleton and Edington[13] to reduce similarly the sensitivity of the bacteria. A combination of oxygen removal and irradiation of suspensions at liquid nitrogen temperature prevents 90 per cent or more of the effect normally produced with irradiation at room temperature in the presence of oxygen. The most plausible explanation for the oxygen- and water-dependent inactivation of bacteria has its foundation in the field of radiochemistry. The complexity of radiochemistry of solutions has been described by the previous speakers, and no attempt will be made here to pinpoint the reactions which lead to the biological damage. It is well, though, to indicate that the evidence accumulated to date is very much in favor of the bactericidal effect of these radiations being in *intracellular indirect* effect, *i.e.*, probably resulting from energy absorption in either the cell fluid, which is mostly water, or in the water layer immediately surrounding the cells.

We have considered, thus far, those systems wherein the physical state of the organism at the time of irradiation has been altered, with resulting modification of the sensitivity of that organism to the radiation. The physiological state of the cell, at the time of exposure of the ionizing radiation, also has a profound effect on that cell's ability to produce a colony after exposure. It has been demonstrated by several investigators that a number of physiological changes take place in a bacterium during the culture cycle, including changes in sensitivity to a variety of injurious agents, both physical and chemical.[15] The conclusion was reached, years ago, that there is a sort of "physiological youth" in growing bacterial cultures, and that young, rapidly dividing cells are the most susceptible to inactivation by these agents. We set up experiments to follow the sensitivity of *E. coli* B/r to X rays during the positive portion of the growth cycle.[10] A large volume of culture medium was inoculated with cells of a known age and, at various times after the inoculation, aliquots of the growing culture were removed. The cells were harvested by centrifugation, washed and suspended in phosphate buffer, and irradiated with a constant dose of X rays. Aliquots of irradiated and control cells were plated on nutrient agar after dilution, and the viable cells were determined by plate count after incubation. FIGURE 1 shows the results of such experiments. The surviving fraction of cells at a constant dose of X rays is plotted as a function of the age of the cells from the time of inoculation. The corresponding phases of the culture cycle are indicated. The phasic nature of sensitivity of the bacteria to X rays is shown in this figure. From the beginning of the lag phase, the cells become more resistant, and the resistance goes through a maximum at the end

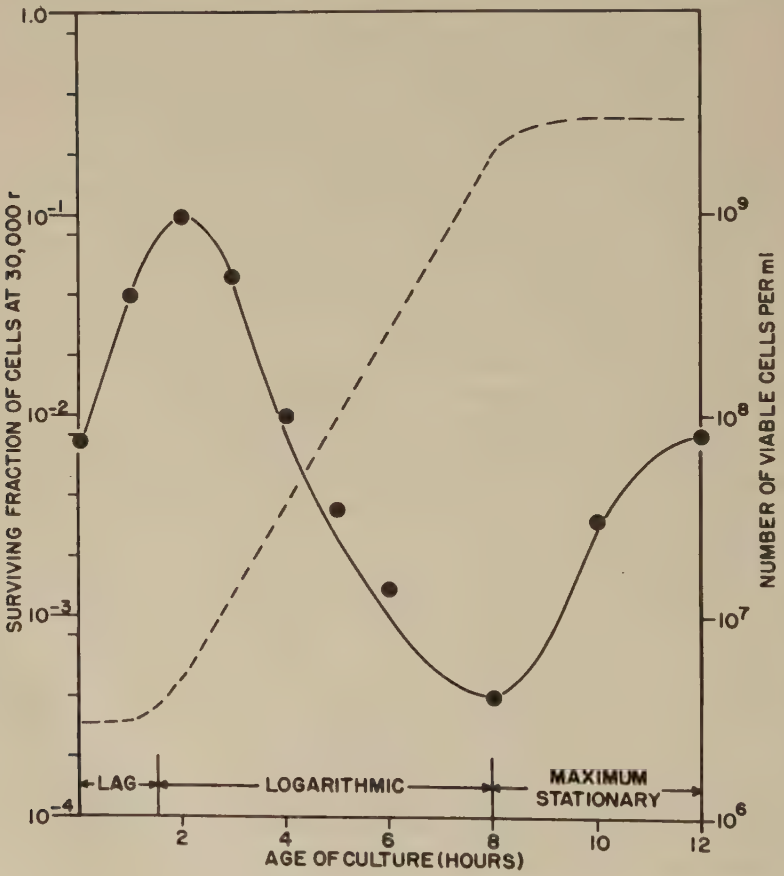

FIGURE 1. Survival at a constant dose of X rays as a function of the age of the culture.

of this phase. Concomitant with the beginning of cell division is a loss of resistance which continues to decrease as the phase of logarithmic growth goes on. The stationary phase is marked by a gradual return to the sensitivity at time zero. FIGURE 2 shows the survival curves as a function of dose for cells at the various times described, and explains, to some extent, the nature of phasic changes of the sensitivity of cells of various stages of the culture cycle. As previously seen, exponential survival curves were obtained for stationary phase cells of *E. coli*. Bacteria taken from the lag phase of the growth cycle give sigmoid curves, showing increasing threshold doses up to the end of this period. It should be noted that all of these curves have similar slopes after the exponential portion is reached. On the basis of the recent microscopic observation of Robinow,[9] DeLamater,[5] Knoll and Zapf,[7] and our own microscopic observations of stained cells during the lag phase, it is tempting to postulate that these curves are first order with respect to the number of nuclear bodies in these cells. It hardly seems fortuitous that the average number of nuclei per cell at this stage and the hit number are about the same. It is well established that, during the lag phase, the total protoplasm—as measured by total nitrogen, enzymatic activity per cell, and nucleic acid content per cell (both DNA and RNA)—increases with no measurable increase in cell numbers. The cells during this stage are large and appear to be either multinucleate or else, as has been proposed by many bacteriologists, they are actually multicellular forms which have not yet divided. The decision as to which of these descriptions is correct depends on further cytological research, and any decisions as to the cause of the multiplicity of hits required to inactivate, as shown in the survival curves for cells in the lag phase, awaits the result of this research. Equally as important as the increased resistance displayed during the lag phase is the rapid decay in resistance which occurs during the logarithmic phase of the growth cycle. This increased sensitivity involves, first, a change back to exponential survival curves (FIGURE 2); cells after several divisions give survival curves with about twice the slope as those at time zero. It is interesting that, here in the bacterial system, there is good evidence for abnormal sensitivity to radiation when rapid cell division is occurring. Mammalian tissues which, in general, are made up of rapidly dividing cells are more sensitive than those in which the mitotic rate is low. If the same principle is involved, we may be able to learn more easily the reason for sensitivity of dividing cells by experiments with microorganisms. It is well to indicate, at this point, that removal of oxygen from suspensions of cells from any stage of growth prior to exposure to X rays brings about the same reduction of the fraction of cells inactivated per unit dose, indicating that the described changes in sensitivity during the growth cycle are not due to variations in the oxygen content of the cells at different stages.

Billen investigated some effects of X rays on the physiology of bacteria and found that irradiated *E. coli* show a normal respiratory rate on a variety of substrates for a short period of time at 37° C.[2] Although the radiation dose was sufficient to reduce the viability to less than 1 per cent, based on ability to produce colonies on nutrient agar, the amount of oxygen consumed by these suspensions for the period of normal respiratory activity indicated that all the

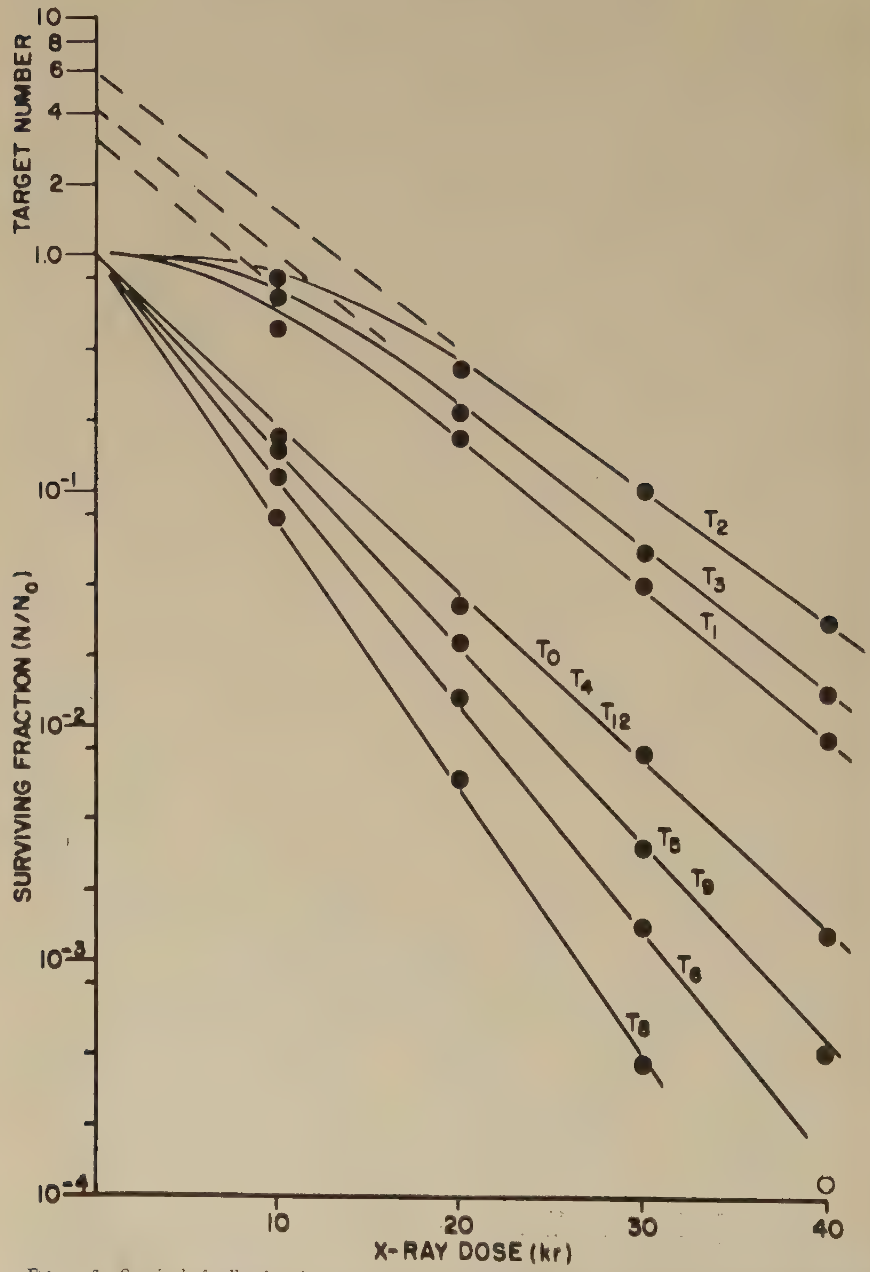

FIGURE 2. Survival of cells of various ages as a function of X-ray dose. The age of the cells is indicated at T_1, T_2, *etc.*, representing cells of 1, 2, *etc.*, hours of age. The target number of the cells is shown by extrapolation of the exponential portion of the survival curves to D_0. Dose rate was 2000 rpm.

cells were participating. After a normal period, a slow but steady decay in the respiratory activity was noted, as shown in FIGURE 3. It is of interest here that a latent period exists before the decay in respiratory activity sets in. In the same figure, we show the oxygen consumption of an identical sample of irradiated cells when the incubation temperature is reduced to 26° C. It is clear that, at the reduced temperature, not only is the latent period extended, but the total oxygen uptake by irradiated cells during this period is greater than at 37° C. It appears that reduction of the incubation temperature may exert a sparing effect on the respiratory system of this organism. Billen[3] has also demonstrated that, although irradiated cells under the previously described conditions can carry out oxidative phosphorylation, as measured by adenosine triphosphate (ATP) synthesis, at 37° C. most of the ATP produced leaks out from the cell and is found in the supernatant. A reduction of the incubation temperature also delays the appearance of this leakage.

We were interested in determining if the reduction of the postirradiation incubation temperature might also affect the viability of irradiated cells by exerting some sparing effect on vital systems.[11] A large number of nutrient agar plates were prepared and equilibrated at various temperatures from 6° to 37° C. Aliquots of an irradiated suspension were then spread on these plates, and they were returned to their respective incubators for a period of 24 hours, after which they were removed to a 37°C. incubator for an additional 24 hours, to allow the colonies to grow up. The plating technique was considered most appropriate for these experiments, since the number of colonies on the plates would be a measure of the number of viable cells under these conditions. The results of such an experiment are shown in FIGURE 4. The surviving fraction of cells at several doses is plotted here as a function of the postirradiation incubation temperature. Although there is no increase in survival at 6° C., at every other temperature up to 37 °C. there is an increase, the survival going through a maximum at 18° C. for this organism. In order to learn something of the rate of this process, which we have called "recovery," we prepared a large number of spread plates and placed them in incubators at different temperatures. At various intervals of time, a set of plates was removed from each of the various incubators, warmed rapidly to 37° C. and incubated thereafter at the same temperature. The results of this experiment are shown in FIGURE 5. Again, the surviving fraction of cells at a constant X-ray dose is plotted, but this time, as a function of the time of holding at each of the temperatures indicated. At 6° C., there is no increase in survival, but rather a slow decrease in the viability of the irradiated bacteria. The rate of increase in survival as measured by the initial slopes of these curves increases with increasing temperature over the range studied. The final survival, on the other hand, goes through a maximum at 18° C. as shown previously. This figure also indicates that holding for 12 hours, at any of the temperatures studied, is sufficient for the recovery process to go to completion. The temperature coefficient for the increase in survival is almost identical to that for growth of this organism, suggesting that a synthetic process is involved in recovery. We can postulate, on the basis of these findings, that survival, as a function of the incubation tem-

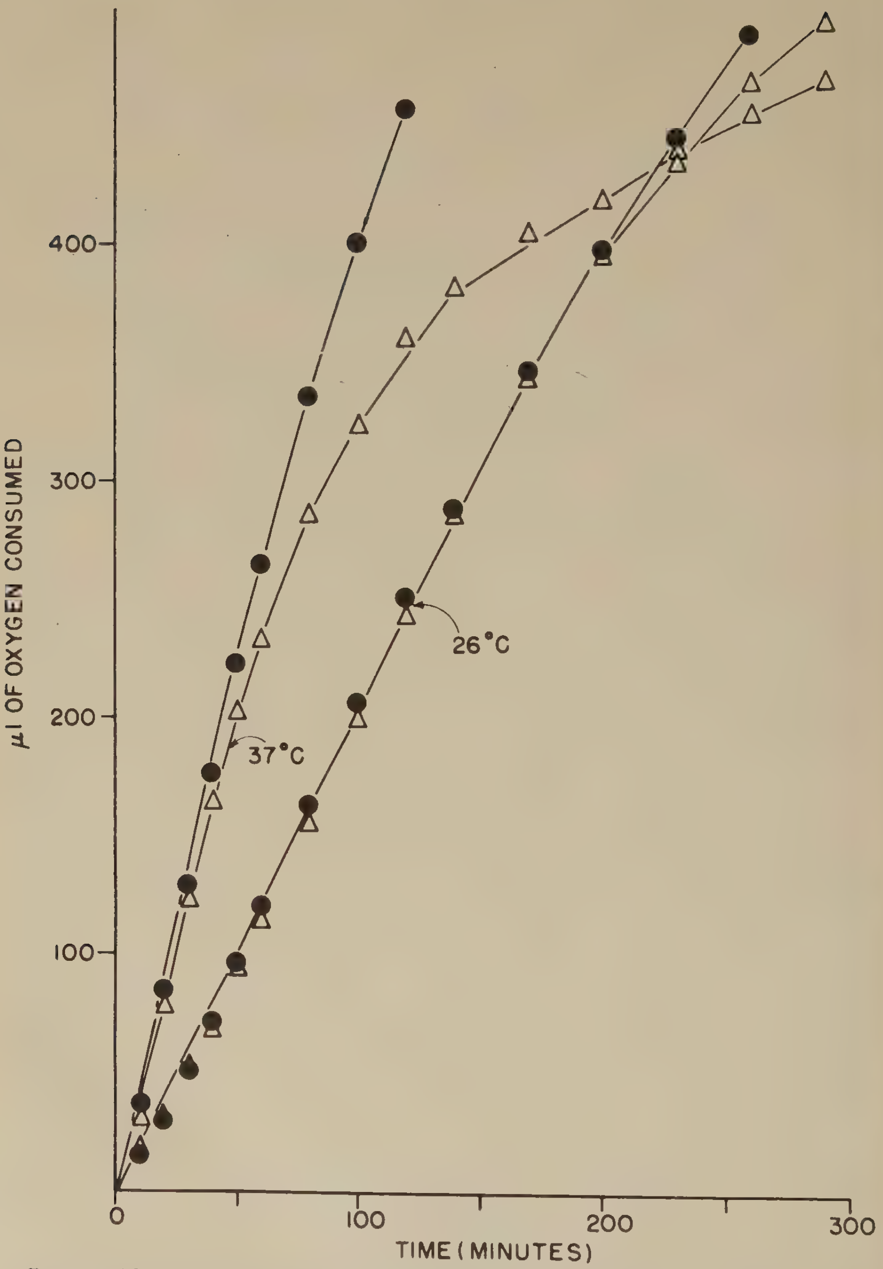

FIGURE 3. The effect of temperature on X-ray damage to bacterial respiration. ● = Controls; ▲ = irradiated cells. (D. Billen, G. E. Stapleton, and A. Hollaender, 1953. J. Bact. **65:** 131.)

perature, represents two temperature-dependent processes. One process, having the same temperature coefficient as growth, is synthetic in nature. Intervention of some destructive process with a higher temperature coefficient occurs at higher temperatures, and the over-all survival at any temperature depends on the relative rate of these two processes. The nature of the destructive proc-

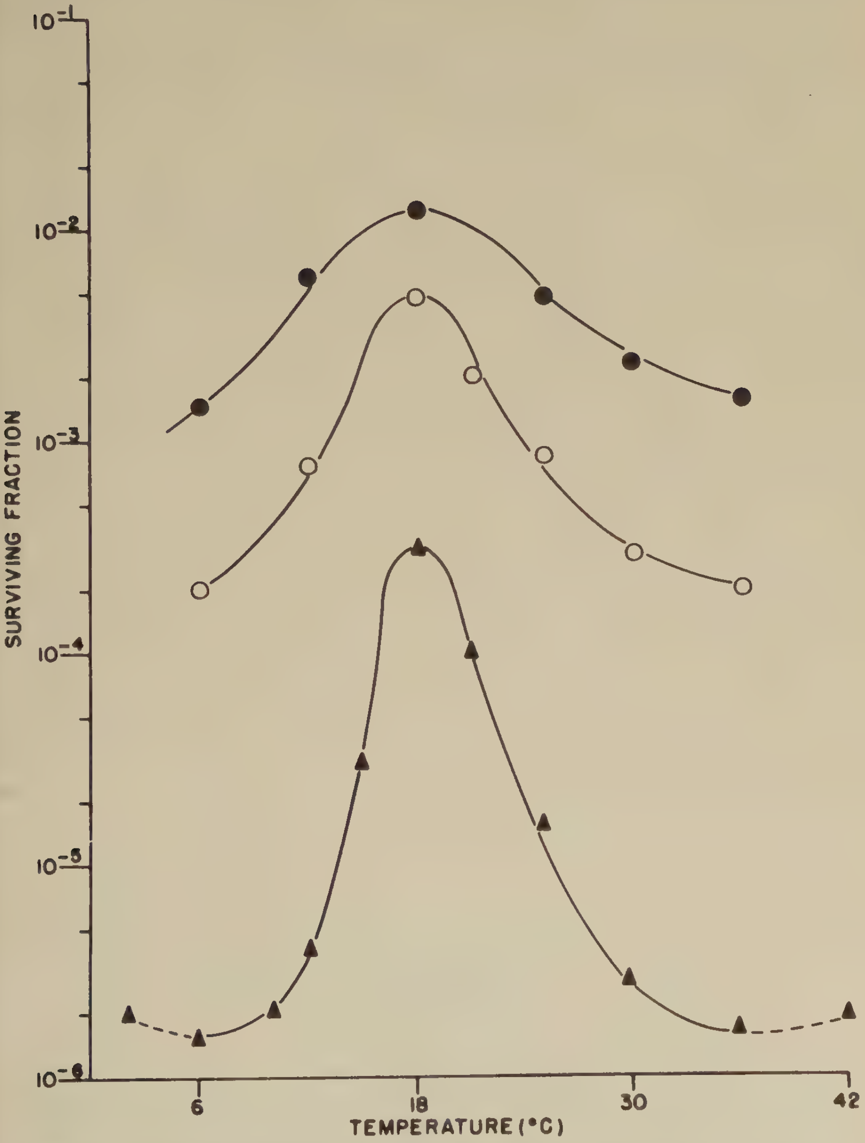

FIGURE 4. Survival at several X-ray doses as a function of incubation temperature. ● = 40,000 r., ○ = 60,000 r., ▲ = 80,000 r. (G. E. Stapleton, D. Billen, and A. Hollaender, 1953. J. Cellular Comp. Physiol. **41**: 344–358.)

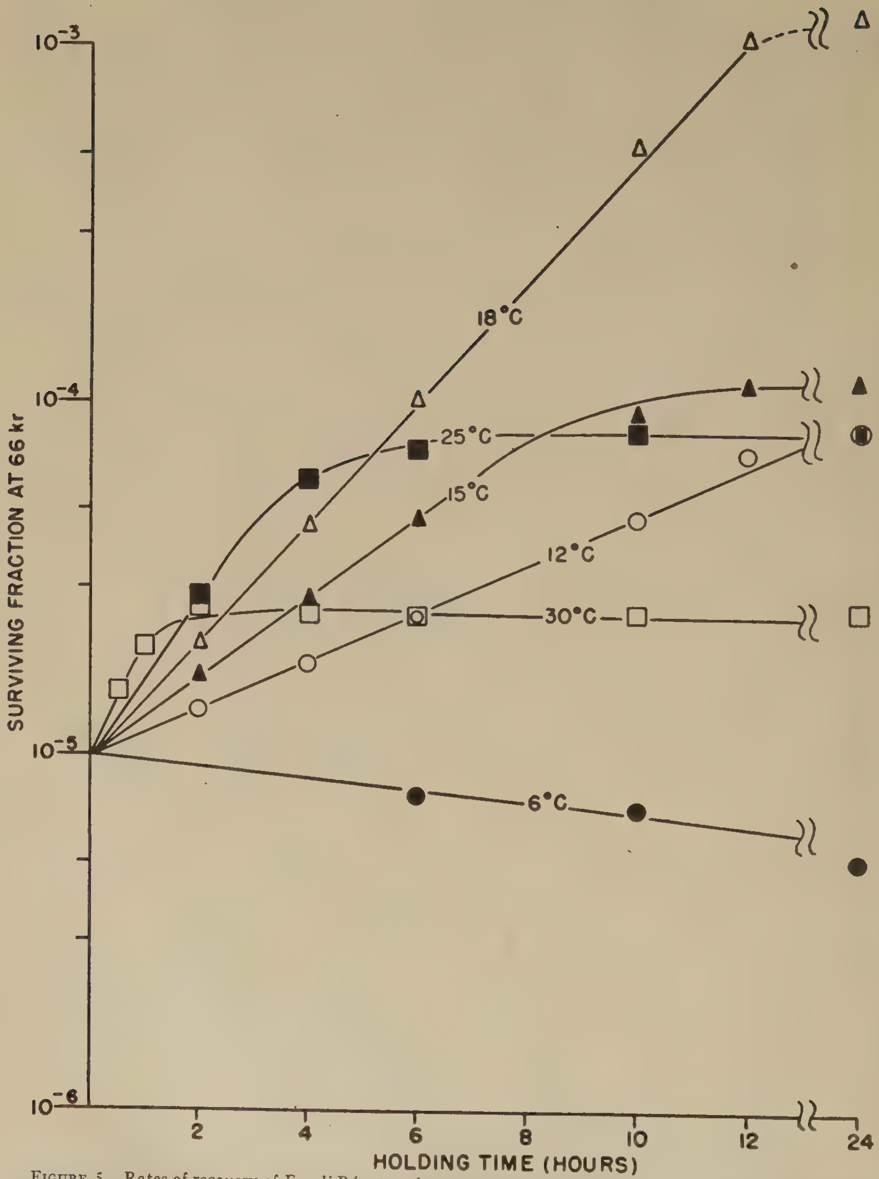

FIGURE 5. Rates of recovery of *E. coli* B/r at various temperatures after 70 kr. of X rays. (G. E. Stapleton, D. Billen, and A. Hollaender, 1953. J. Cellular Comp. Physiol. **41**: 344–358.)

ess is not suggested by these experiments, but may involve an induced increased thermolability of some vital system in this bacterium. The work of Clark[4] indicated that radiation may sensitize protein to denaturation at lower temperature. Whether *E. coli* protein, especially enzyme protein, is sensitized by radiation can be learned by direct measurement. Experiments to test this possibility are anticipated in the future.

Survival curves for *E. coli* incubated at 37° and 18° C. are shown in FIGURE 6. It is clear that reduction of the postirradiation incubation temperature brings about a reduction in the slope of the survival curve. The dose of X rays required to inactivate the same fraction of cells at 37° C. is about 60 per cent of that at 18° C. In other words, about 40 per cent of the radiation effect may be reversed by incubation at the lower temperature. We were interested in determining whether or not the process we call recovery is an endogenous process similar to "photoreactivation," or whether some of the metabolites contained in the nutrient agar were required. Since unirradiated *E. coli* grows very well

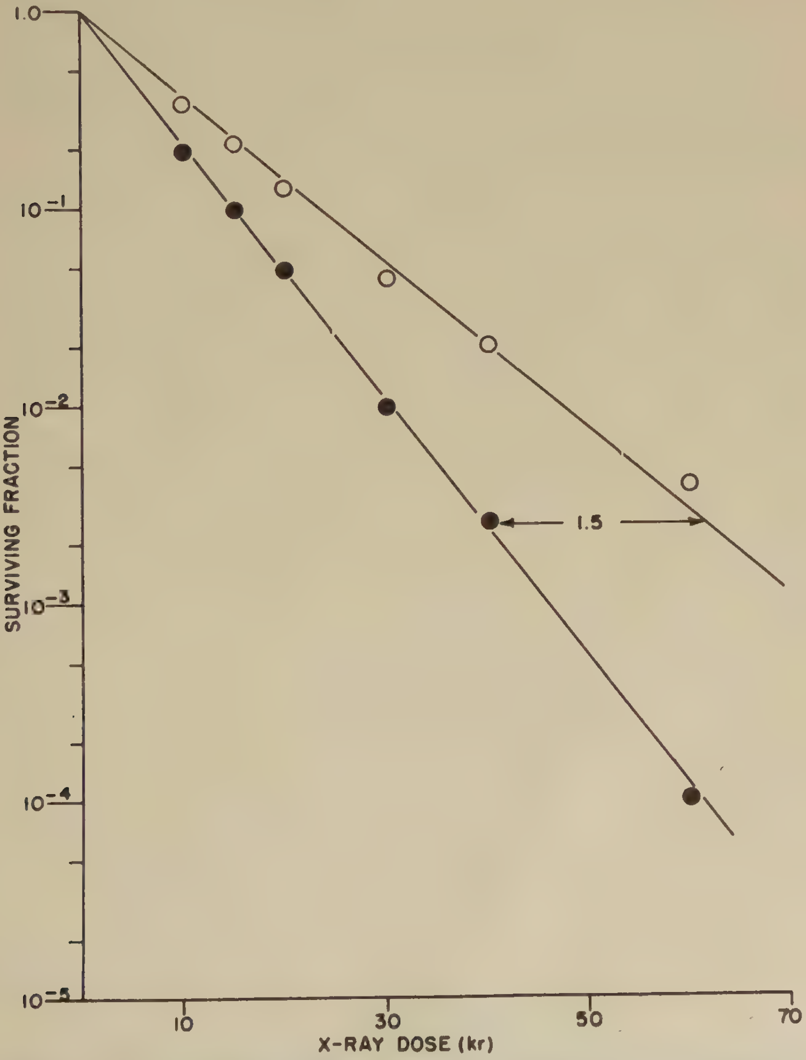

FIGURE 6. Recovery at various X-ray doses *E. coli* B/r. ○ = holding at 18° C, ● = holding at 37° C. (G. E. Stapleton, D. Billen, and A. Hollaender, 1953. J. Cellular Comp. Physiol. **41**: 344–358.)

on synthetic media composed of inorganic salts and glucose, a comparative study was carried out on the effect of incubation temperature on survival of irradiated cells on synthetic and complete media such as beef extract, yeast extract, or various other tissue extracts. The results of these studies are shown in FIGURE 7.[14] The upper curve shows the survival at a constant X-ray dose for cells plated on complete media, in this case yeast extract, and is essen-

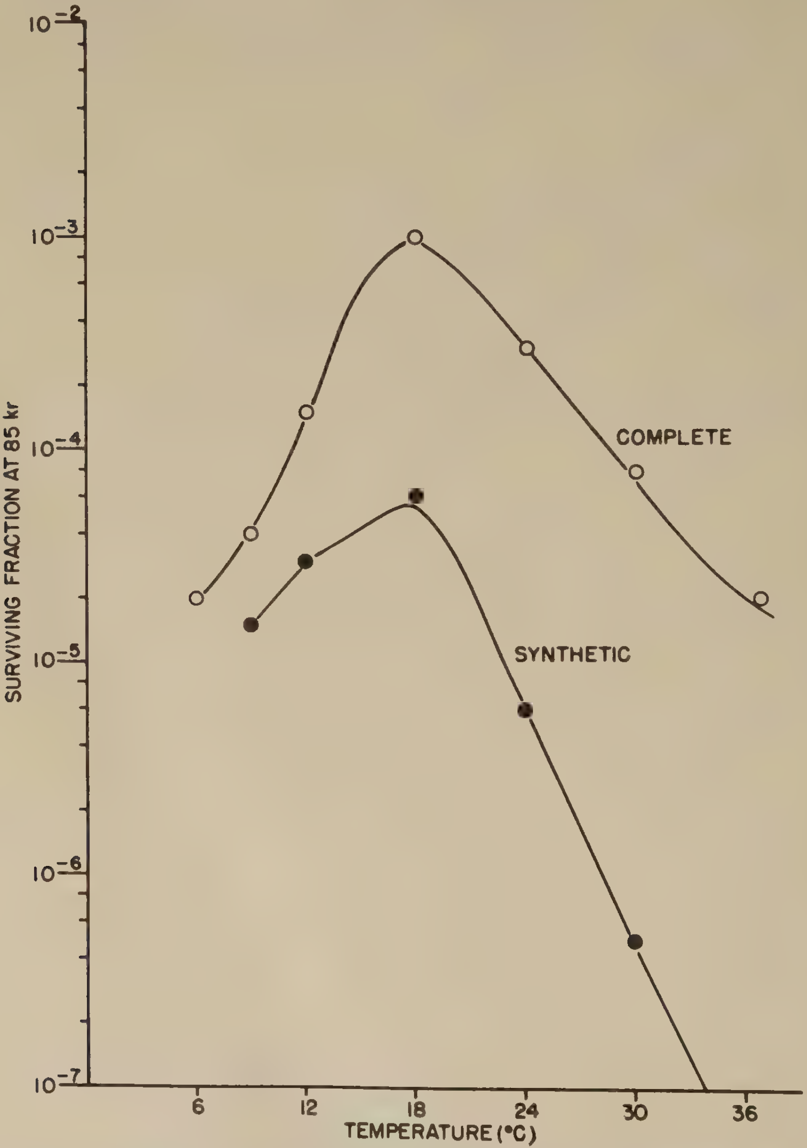

FIGURE 7. Surviving fraction at 85 kr. gamma rays for *E. coli* B/r incubated at various temperatures on synthetic medium compared with that obtained on complete medium (Difco nutrient agar).

tially similar to those shown previously. The lower curve shows the results obtained when a synthetic medium was used. Very little increase in survival is obtained over the temperature range from 6° to 18° C. as compared with that obtained on a complex medium. Moreover, the decrease in survival above 18° C. is extremely large as compared with that obtained on complete media. One might say that the sparing effect of reduced incubation temperature is much more pronounced when irradiated cells are plated on minimal media. Also, there appears to be a stimulatory effect of some components in the complex media at all temperatures, but it is most pronounced at the higher temperatures. On the basis of these findings, we have attempted to determine what factors contained in complex biological materials will stimulate recovery in this strain of *E. coli*. An assay has been developed, based on the relative increase in survival on the synthetic or basal medium plus additives, as compared with that on basal alone. The assay for various concentrations of yeast extract added to basal medium is shown in FIGURE 8. As can be seen here, a linear relation exists between the increase in survival over basal and increasing concentration of yeast extract, from a few micrograms up to greater than 20 mgm. per plate.

A number of sources of recovery factors have been investigated. Assays have been made of crude homogenates and of hot water extracts of various tissues, and it was found that the water-soluble fraction contains most of the activity of the homogenate. The relative activity of hot water extracts of several tissues of the rabbit are shown in FIGURE 9. It is of interest here that the hot water extract of the spleen is an outstanding source of the factor or factors.

Heavy metal precipitation of hot water extracts of spleen, as well as yeast extract, have resulted in poor resolution of the active material, with apparent

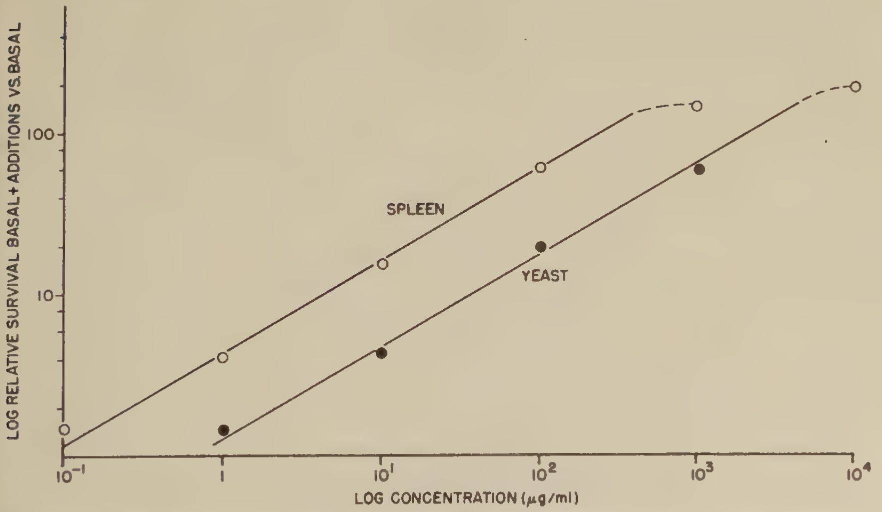

FIGURE 8. Relative activity of spleen (EtOH extract) and yeast extract plate assays with incubation at 37° C.

loss of activity. Hydrolysis, either acid or alkaline, with or without autoclaving, has not altered the activity of crude extracts. The active material is readily adsorbed on charcoal and can be removed in ammoniacal ethanol, with about fivefold purification. Approximately the same purification can be obtained by extraction with 90 per cent ethanol. Further purification can be obtained by extraction with 95 per cent ethanol or acetone. Large volumes of alcohol or acetone will precipitate the activity. About twentyfold purified material may be obtained by this technique. The resulting material is still too crude to permit resolution of the activity. Paper chromatography of either crude or purified fractions indicate that there are several partially active materials which appear to act synergistically in promoting recovery. Paper chromatography, with the butanol water system at various pH's, indicates that the most active fractions have acidic groups. Counter-current extraction with butanol-water has given similar results. These findings have given us reason to suspect that we are working with a multiple factor, and that purification with respect to a single requirement may have been misleading in earlier attempts at purification. Simultaneously with these purification attempts, we have endeavored to construct a complex synthetic medium which might substitute for the natural materials. To date, the known vitamins, as well as numerous growth factors when added to basal medium, are not stimulatory in the recovery system. Casein hydrolyzate, as well as a simulated synthetic mixture of the amino acids, gives some stimulation. Addition of a mixture of vitamins and growth factors to the amino acids mixture has not given any ac-

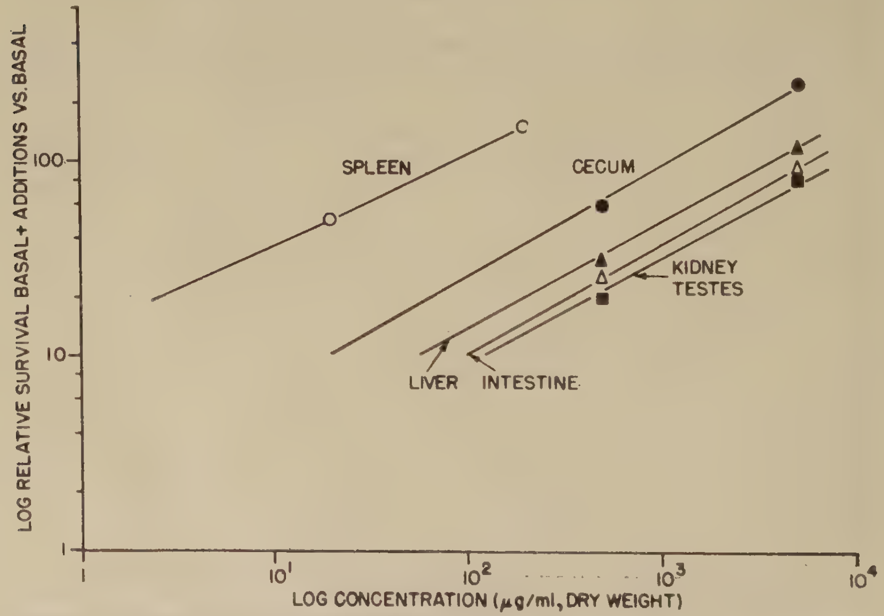

Figure 9. Relative activity of various rabbit tissue homogenates in increasing viable cell count of gamma-irradiated *E. coli* with 37° C. incubation.

tivity over that obtained with the amino acids alone. On the basis of the information obtained from paper chromatography and by studying combinations of amino acids, we have been able to resolve the amino acid activity to four amino acids: glutamic acid, serine, methionine, and tryptophane. These four amino acids give the same stimulation which can be obtained with casein hydrolyzate or the synthetic mixture. We were anxious to determine if polypeptides would be more effective than amino acids. A series of dipeptides, as well as a phosphopeptone of about 18 amino acids, about a third of which was glutamate, was assayed. The results of these experiments indicated that glutamyl dipeptides were the most active, but they were no more active than their component amino acids. A mixture of purine and pyrimidine bases, added to the amino acid mixture, gave more activity than the amino acids alone. Some of the media designed for growth of heterotrophic organisms have been active in the recovery system, and the activities on the components of these media are being resolved at present in our laboratory. The results will be reported at a later date. Preliminary results of experiments in which modified *Lactobacillus* assay media have been used indicate, again, amino acid and purine and pyrimidine requirements. These findings appear, at present, to implicate the involvement of more than one system in the recovery process, as far as this strain of *E. coli* is concerned. Much more work will be required before a mechanism can be proposed for the recovery process in bacteria. It is hoped that some of the findings of these experiments may help us achieve a better understanding of recovery in higher forms of life and, ultimately, will help us to gain further insight into some metabolic systems involved in radiation damage of living cells in general.

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RADIATION EFFECTS IN *PARAMECIUM*

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The use of homogeneous populations of cells in studies for exploring the basis of radiation damage in living systems has contributed significantly to radiobiological knowledge. Among others, bacteria, protozoa, fungal spores, yeast cells, bacteriophage and other viruses, tissue cultures, isolated tumors and other animal cells, and, in certain instances, *in situ* tissues in intact metazoan constitute biological systems whose *milieu* can be controlled with reasonable accuracy, and the treatment of which can be given under known and sometimes controlled conditions. In such systems, in which interactions among radiation-induced phenomena occur principally at an intracellular level, certain of the responses to a single kind of radiation treatment can be separated and measured accurately, in contrast to the situation in mammals; for instance, in which interactions must occur at both intercellular and intracellular levels.

Paramecium became especially useful for radiation studies following the significant work of Sonneborn[38] and his colleagues, for there was promise of distinguishing in the one cell between radiation effects which are "vegetative," in the sense that the primary action of the radiation is on nongenic structures; and "genetic" effects, in the sense that the primary action is on the heredity determiners. Following irradiation, these three effects, at least, can be conveniently measured: (1) the death of the cell; (2) the disturbance of the cell division mechanism, which we call suppression of fission; and (3) an effect which, because of its appearance only after the self-fertilization process called autogamy, is very likely an expression of damage by the radiation to the micronuclei and is, therefore, a genetic effect. In addition, the structural changes of the cytoplasmic elements and of the macronuclear and micronuclear structures are subject to direct examination, and reference to the three effects noted above is possible. It is possible to treat an animal in a variety of ways under several kinds of conditions before, during, and after irradiation and search for differences in the nature of the various responses, in the hope of isolating and separating them.

This paper will review briefly the effects of X rays on *Paramecium aurelia*, stock 51, mating type VIII, kappa containing, in regard to these three phenomena: death of cells, death after autogamy, and suppression of fission. It will relate postirradiation temperature changes to expression of these effects, and will attempt to correlate these results with those obtained by others after X rays.

Death of cells. Several investigators have reported on the lethal effects of X rays in *Paramecium*.[4, 18, 33] Powers and Shefner[33] obtained a sigmoid response curve with the LD_{50} at approximately 250,000 r. Animals in these experiments were starved in bacteria-free salt solution for 12 to 14 hours before exposure, in order to eliminate the differences in response which might be expected from cells in different stages of the division cycle. Although starvation induces autogamy, these cells had not been starved for the length of time necessary to induce the phenomenon. The same procedure was used in later experiments[32] reviewed here, which were designed to measure the effect of postirradiation temperature on the incidence of death in the first and second division generations after irradiation. Except for the period of irradiation, all experiments involving effects of temperature after irradiation on *Paramecium* were carried out in temperature-controlled rooms.

FIGURE 1 shows the effect of postirradiation temperature on survival (X rays at 14,000 r. per minute, measured with an air ionization chamber, at 50 kvp. from a Machlett OEG-60 tube through 0.25 mm. Al, with resulting H.V.L. of 0.16 mm. Al, or a 90 per cent emergent dose with our arrangement). Results are presented as accumulated death up to the third day after irradiation for two temperatures. The 50 per cent survival dose at the higher temperature (32.5° C.) was 200,000 r., and at the lower (17.5° C.), 260,000 r. Another

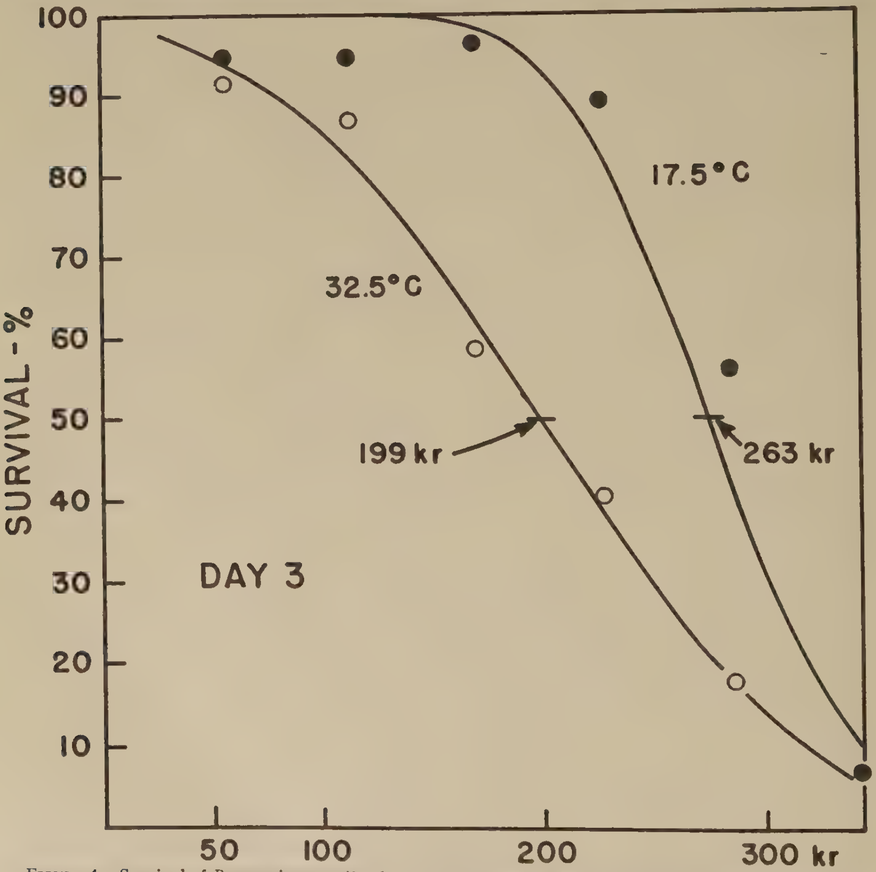

FIGURE 1. Survival of *Paramecium aurelia* plotted against dose at two postirradiation temperatures cumulated through day 3. The numbers indicate LD_{50} dosages at the two temperatures estimated visually from probit plots.

experiment carried out at 24.5° C. was omitted from the figure. At this intermediate temperature, intermediate survival was noted.

The effect of low temperature in increasing postirradiation survival is obvious. Unfortunately, because of the difficulty of control survival at these temperatures, it was not possible to test survival below 16° C. to see if there is a temperature of maximal survival at an intermediate post treatment temperature, suggested by the data of Schrek[37] and later demonstrated by Stapleton *et al.*[40] It is conceivable that the survival noted at 17° C. is the maximum that can be expected after X rays.

These results agree, in general, with those of others after X ray (see below), but may be different from those observed after ultraviolet treatment. In *Escherichia coli* B, survival after ultraviolet irradiation increased as the temperature increased to 44° C. (Stein and Meutzner[41]), and from 30° to 40° C. (Anderson[2]). It has also been observed that survival is less at 15° than at 37° C. (Berrie[6]), but it is temperature independent from 15° to 25° C. (Sacher[35]). In *E. coli*, then, after UV exposure, survival appears to decrease as postirradiation temperature decreases from 44° to 25–15° C. However, the situation in "diploid" yeast cells may be different (Latarjet[25]), for survival after either UV or X irradiation is increased two to three times at 5° C. over that observed at room temperature.

After X irradiation, survival is known to be related to post treatment in the following ways. Cook[8] observed that fertilized *Ascaris* eggs, when stored at 5° C. for seven weeks following exposure to X ray (5,000 r.) and then returned to 25° C., developed successfully in 40 per cent of the cases, whereas only 2 per cent of the eggs maintained at 25° C. developed successfully. Rugh[34] reports similar results when zygotes derived from irradiated sperm of *Arbacia* were maintained for 24 hours at 10° C. Development to ciliated trochophores occurs in 50 per cent of the embryos fertilized by sperm receiving 65,000 r. and held in the cold, while only 40 per cent develop after 35,000 r. when fertilization and development is allowed to proceed at higher temperatures immediately after irradiation.

In the bacteriophage S13, it has been shown recently (Alper[1]) that there is no hypersensitivity to heat after UV or X irradiation. Although it appears that there are no temperature-dependent postirradiation processes leading to inactivation, the sensitivity of the bacteriophage to reducing substances and to H_2O_2 suggests that some reactions do occur following X irradiation.

Stapleton[40] and his colleagues have demonstrated that survival in *E. coli*, after X irradiation, depends on the postirradiation temperature, with a peak for highest survival in the 15° to 25° C. region, depending upon the bacterial stock used. This effect occurs, however, only when the bacteria are grown in nutrient broth, and not when they are grown in a minimal medium. It therefore appears to depend upon the presence of particular metabolites. (In this connection the results of Ducoff with *Tetrahymena*[9] and *Chilomonas*[10] are pertinent and are mentioned below.)

In general, it appears that, following exposure to UV, survival decreases as postirradiation temperature decreases, with the exception of yeast, in which there are more survivors at 5° C. than at higher temperatures. After X ray, the general relation is reversed, for now survival increases as postirradiation temperatures decrease to an optimal low temperature in at least one and, perhaps, all cases.

Giese and Heath[18] observed lowered survival when Paramecia are exposed, after X ray, to a temperature shock of 42° C. for 1.5 minutes. They report that recovery from this hypersensitivity occurs when the animals are fed. While we have no measurements on this kind of recovery, we do observe that an increase in postirradiation temperature from 17° to 32° C. results in a steadily decreasing survival. This finding is consistent with the observations in other microorganisms cited above. The mechanism is not obvious from the observation, but it is suggestive that most of the deaths observed at the highest temperature was immediate: lysis occurred on transferring the cell into warm culture fluid. Lysis was observed less frequently at the intermediate, and least frequently at the low temperature. This effect appears to be membranous or ectoplasmic—at high temperatures, the membrane, weakened by radiation, does not remain intact, and the cell dies. While other explanations are conceivable, the phenomenon is probably distinct from the delay in cell division which is seen at the same time, and any explanation should attempt to account for differential recoveries of different systems. All the survival data of Stein and Meutzner, of Anderson, of Berrie, of Latarjet, and of Stapleton and Giese can be interpreted as effects distinct from the fission-suppression phenomenon and, while "recovery" of the cells from this damage could be measured, it has not been done completely enough to allow this result to be equated at the present time with recovery in cell-division processes.

Effects on mutations. The question as to whether the recognizable genetic effect can be modified by changes in postirradiation temperatures is of importance when distinction of modes of action is attempted. An effort was made to measure this modification by noting the effect of postirradiation temperature on death following autogamy. After incubation in salt solutions overnight, cells were given doses of 4, 8, and 12 kr., and either transferred immediately to 31° C. or maintained at 25° C. The cells were kept at these temperatures throughout the time of preautogamous division, autogamy, and postautogamous incubation. The results are equivocal, because the room temperature controls did not show the effect expected on the basis of past experiments.[33] We may nevertheless note that, at the three dosages, 20 per cent, 35 per cent, and 30 per cent, postautogamous death was observed at 31° C., while 30 per cent, 50 per cent, and 30 per cent was observed at room temperature. The room-temperature results were generally higher (more death). It must be carefully noted here, however, that this is a rather variable response—the magnitude of the difference being on the order of the replicating error and, in the absence of a very clear-cut difference, we must conclude that the postirradiation temperature has not been shown to have an effect on the postautogamous effect of irradiation.

Any interpretations of the *Paramecium* data concerning this "genetic" effect must be qualified by the uncertainty concerning the real nature of postautogamous death. In this system, a micronucleus is irradiated and under-goes meiosis with "sexual" fusion of pronuclei, giving rise to a new zygotic nucleus which is homozygous. The presumption is that death after this reorganization is due to genetic lethal changes, either dominant or recessive, which were brought about by the treatment. Kimball[22] gives some evidence concerning the number of genetic events necessary for a given amount of postautogamous death, but the evidence is not decisive, and no measure has been made of the extragenic contribution to lethality at autogamy. That such a relationship may exist is indicated by Geckler[15] and by our own experience.[30]

The evidence here indicates little effect of postirradiation temperature on postautogamous death. This kind of result is seen in this temperature range for X-chromosome lethals in *Drosophila*. At the very low end of the physiological range, however, it has been demonstrated by several investigators (Muller,[28] Medvedev,[27] and King[24]) that, after irradiation in the cold, the frequency of sex-linked lethals in *Drosophila* is higher than that observed after irradiations at room temperature and, recently, Baker and Sgourakis[5] showed that the effect is due partly to increased O_2 concentration in the tissues at the low temperature. But none of these authors indicates that *post*irradiation temperature has any influence on the induced mutation rate. In barley, Caldecott and Smith[7] report a result in the opposite direction, *viz.*, treatment at 85° C. after X irradiation of barley seeds increases the mutation frequency.

In bacteria, several kinds of effects are noted. After irradiation, the number of "mutations" observed may show a maximum at a relatively low postirradiation temperature—18° C. for *E. coli* (Anderson and Billen[3]) after X ray, and 19° C. for *S. typhimurium* (Miller and Plough, 1952, in Latarjet[26]), after UV, both measuring biochemical back mutations. Berrie,[6] however, measuring reversion to streptomycin dependence in *E. coli*, and Witkin,[42] noting changes to T-1 resistance, show that at approximately 15° C. the number of mutations is much lower in both instances than at 37°C. after ultraviolet irradiation. The effect on T-1 resistance is thought to be changeable up through the first postirradiation cell division, and this evidence is taken by the author as indication of an indirect action of UV on the system determining phage resistance.

In bacteria, then, after UV treatment, reduction of temperature may increase the frequency of mutations observed, or it may decrease it, while the only experiments testing effect of temperature after X irradiation (Anderson and Billen[3]) show an increase in mutation frequency to a maximum as the temperature decreases, much as bacterial survival is affected by postirradiation temperatures (Stapleton, *et al.*[40]). No such temperature dependence is apparent in the *Paramecium* studies. In attempting explanation and reconciliation of these results, however, one should remember that, difficult as it is to interpret the lethal postautogamous results in *Paramecium* as an effect on genetic determiners in the *Drosophila* sense, it is more difficult to interpret these changes in bacteria as mutations of the same sort and, perhaps, alternative interpretations of the nature of some of the changes themselves may explain more successfully the radiation results.

Suppression of fission. Our early results[33] on suppression of fission by X ray was followed by the careful analysis by Kimball *et al.*[23] of the pattern of fission times during successive cell divisions after UV, X-ray, and HN2 treatment.

We have extended the observations further with a more complete analysis of the results,[36] and the effects of postirradiation temperatures on the general pattern of response[32] have been measured. In these experiments, single irradiated cells were isolated into separate culture dishes, numerous cells for each treatment group. Observations on the number of fissions occurring in each culture dish were made after the first 24-hour interval after irradiation, and then one daughter cell was transferred to a new dish. After the next 24 hours, another count was made. In the first experiments this procedure was continued for four days, and, in the most recent experiments, for five days. The mean number of fissions of the treatment group divided by the mean number of fissions observed in a series of untreated cells was taken as the relative division rate for the particular treatment group on that particular day. It is to be noted

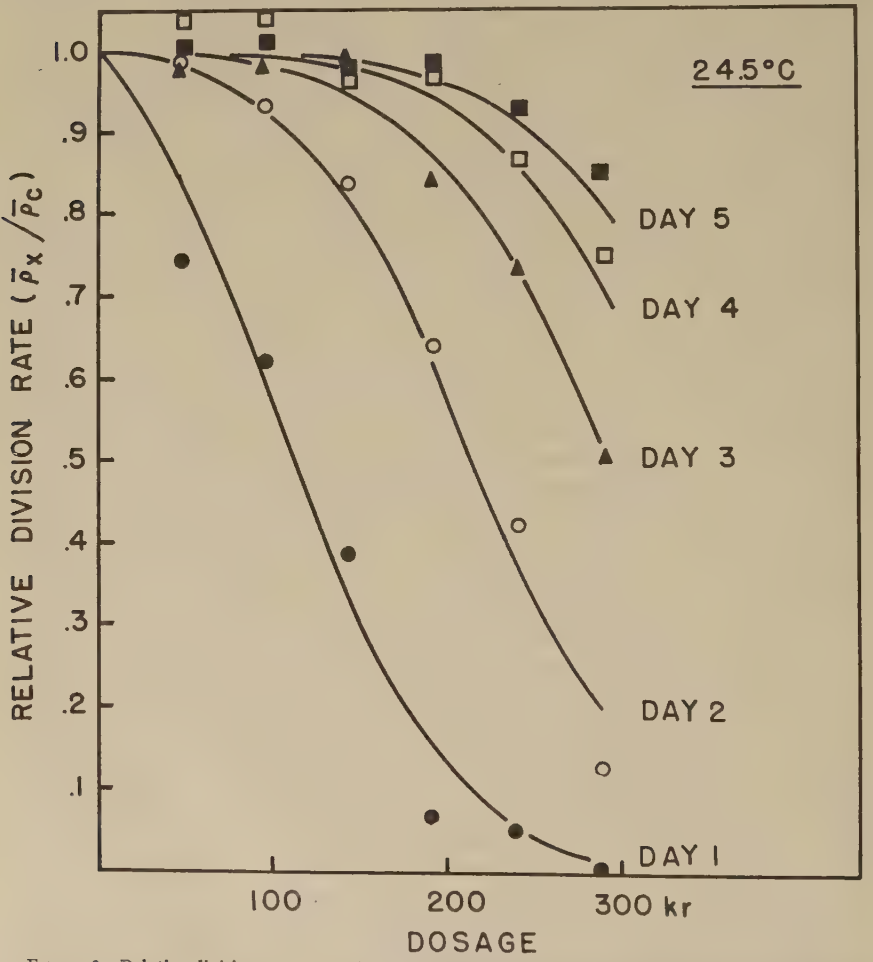

FIGURE 2. Relative division rates on each of five postirradiation days at 24.5° C as affected by indicated dosage of X rays.

that this method of stating suppression of fission as a percentage of the control rate is different from that employed in the study of effects of HN2,[31] in which hours delay beyond the control in reaching control fission rate was used as the measure. Kimball *et al.*[23] used essentially the raw fission rate which was compared to the control graphically rather than numerically.

In FIGURE 2, the relative division rates of X-irradiated animals are presented for each of the five postirradiation days covered by these observations. Each point is an average derived from more than 20 animals. It is seen that, on day one, suppression of fission is complete at approximately 290,000 r., approximately the dose at which 50 per cent of the exposed cells do not survive at this temperature. The form of the curve presented—the logistic—was chosen for reasons to be presented shortly. On the second day, recovery is obvious, al-

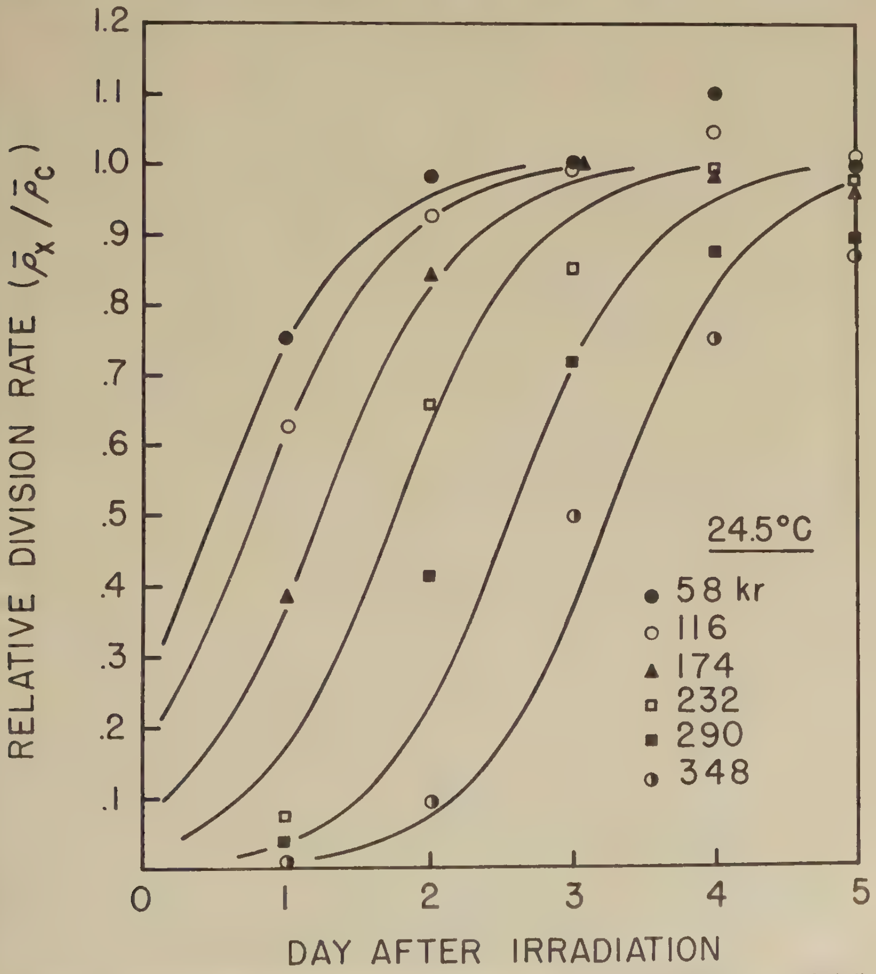

FIGURE 3. Relative division rates of cells on five successive days after X irradiation at 24.5° C. at the indicated dosage levels.

most all of the exposed cells now dividing. On day three, the 175,000 r. group is back at control rate; on days four and five, fewer groups are dividing at rates slower than the untreated controls. Recovery time increased as dosage increased, and over this exposure range no permanent damage to the fission mechanism was observed.

The gradual recovery was demonstrated by each clone during the period of observation. The means obtained were not the result of some cells dividing at control rate while others failed to divide, but were reflections of intermediate rates observed in all the treated clones.

The time dependence of recovery at a particular dose level is illustrated in FIGURES 3, 4, and 5. At each temperature, recovery is approximately linear in the measurable recovery range. The effect of high dosage is to lower fission

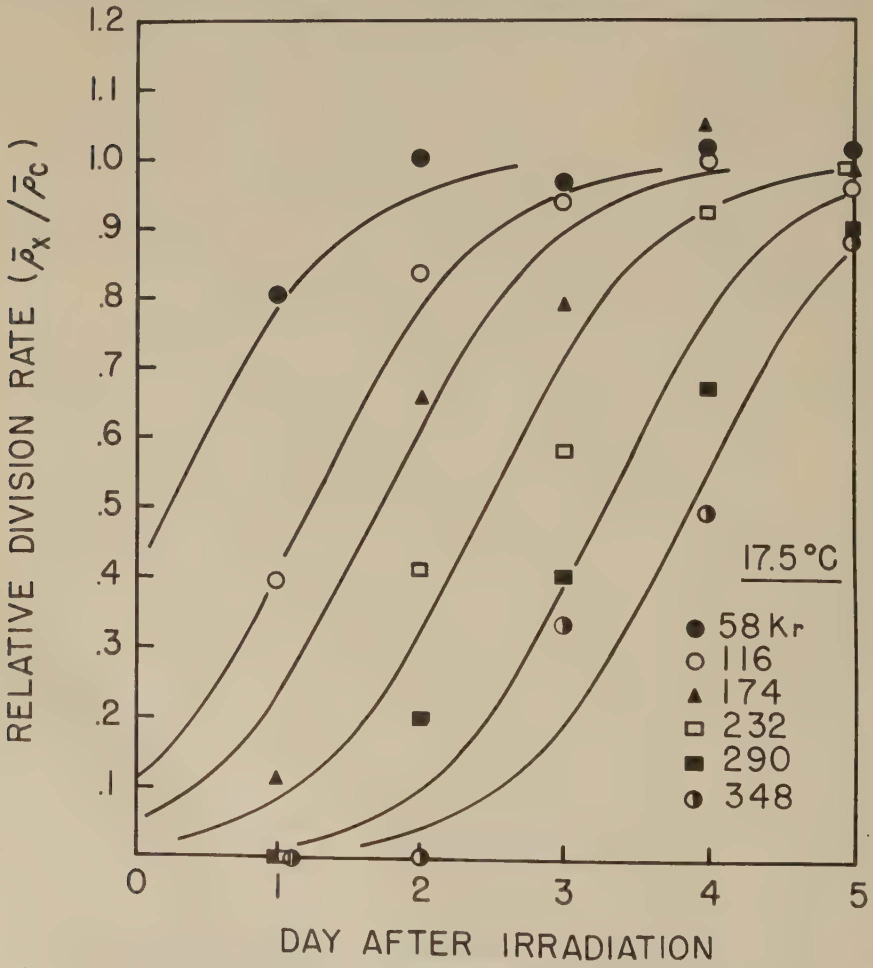

FIGURE 4. Relative division rates of cells on five successive days after X irradiation at 17.5° C. at the indicated dosage levels.

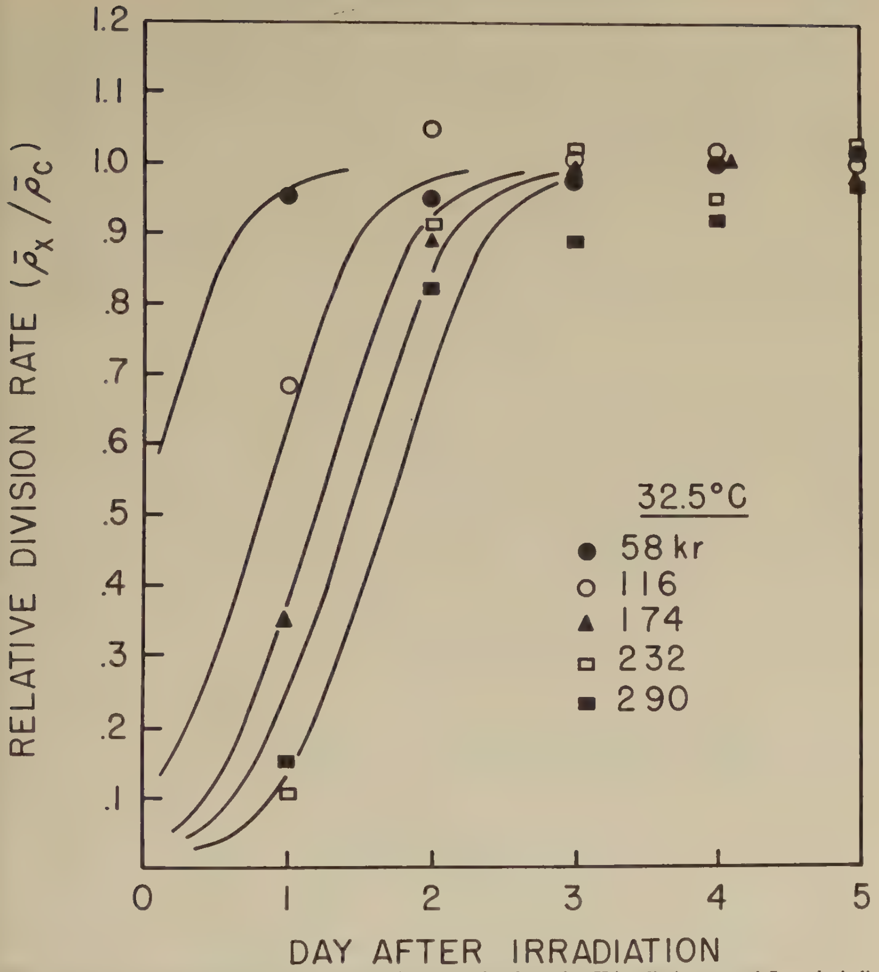

FIGURE 5. Relative division rates of cells on five successive days after X irradiation at 32.5° C. at the indicated dosage levels.

rate. Since all recovery slopes after different dosages at each temperature appear the same, an independence of recovery rate and the total dose (within these dosage ranges) is suggested.

The use of the logistic is not required by either of the two methods of expressing the time-dose relationship. However, it does fit the data at least as well as one other possible method of expression, namely the probit, and it has the advantage of being conceptually meaningful, suggesting a mode of action of radiation amenable to experimental manipulation and test. We assume that division rate depends on the concentration of particular cellular materials which are exponentially inactivated by radiation as

$$N = N_s e^{-mD} \tag{1}$$

where N_s is the number of particles necessary for normal division, and N is the number after dose D. The restitution of the particles to normal number is assumed to be an autocatalytic process, with no implications at the present time intended concerning the actual manner of production. The rate of restoration after a radiation dose is

$$\frac{dN}{dt} = kN(N_s - N) \tag{2}$$

N being the number at time t. The solution of EQUATION (2) using (1) to evaluate the constant of integration is

$$\frac{N}{N_s} = \frac{e^{kt}}{e^{kt} + e^{mD} - 1} \tag{3}$$

which describes the time course of the increase within the cell of the number of particulates after irradiation. The relative fission rate, ρ/ρ_s, must further be assumed to vary with number of particles N in a nonlinear manner so that the fission rate is proportional to the number of particles for low N, but increases at a progressively slower rate as N increases. It is not yet possible to state the explicit kinetics of this relation, but we have found that the empirical relation

$$\frac{\rho}{\rho_s} = \frac{N}{N_s} \frac{(1 + a)}{\left(\frac{N}{N_s} + a\right)} \tag{4}$$

fits the data well and is convenient to apply. Introducing EQUATION (3) into (4) we find the equation for the kinetics of fission rate

$$\frac{\rho}{\rho_s} = \frac{1}{\frac{a}{1 + a}(e^{mD} - 1)e^{-kt} + 1} \tag{5}$$

This is the equation used to describe the data. The constant k is the restoration constant of the particulates (or some kind of mean if there are several types with different restoration rates) at this temperature, and m is the inactivation constant.

It is proper to repeat here that the rate of restitution which results in recovery of the cell is not dependent upon the amount of damage suffered in these dosage ranges, provided the cell survives. It would be of considerable importance to ascertain definitely whether there is causal relationship between this system and the immediate death of the cell following radiation. The evidence at hand now indicates that there is none.

The effects of incubation[32] after irradiation at varied temperatures are illustrated by FIGURES 3, 4, and 5. In FIGURE 3, the recovery course observed at 24.5° C., during the five days following irradiation after the six dosages, is shown. The logistic assumption is used, the data being generally described by a family of curves with the same slope, with dose determining the depth to which the fission rate is depressed. When the temperature is changed to

17.5° C. (FIGURE 4), the same description is valid, but now the curve is more shallow, while at 32.5° C. (FIGURE 5) the curve is much steeper. Behavior at each of the conditions of culture is the same, except for the change in slope.

The data suggest that the recovery process may be associated with the fission process. Furthermore, the observation of Kimball[23] that recovery is complete after six to eight cell divisions, after several kinds of treatment, implies a connection that is not proved by the data as they stand. Accordingly, inquiry was made into the relationship between extent of recovery and number of cell divisions completed, and in the following way. The recovery function gives the number of fissions completed after time t as

$$F = \int_0^t \left(\frac{\rho}{\rho_s}\right) dt \tag{6}$$

which evaluates as

$$F\left(\frac{\rho}{\rho_s}\right) = -\frac{\rho_s}{k}\left[\ln\left(1 - \frac{\rho}{\rho_s}\right) - \ln\frac{a(e^{mD} - 1)}{1 + ae^{mD}}\right] \tag{7}$$

in which ρ/ρ_s has the former meaning of relative fission rate—a function of N/N_s. This expression says that a plot of $\ln(1 - \rho/\rho_s)$ against the number of fissions completed $F(\rho/\rho_s)$ should yield a straight line with the slope $-\rho_s/k$. The constant k is the restoration constant evaluated with the original logistic expression. This constant changes with temperature, and the slopes of these straight lines might be expected to change with temperature.

In FIGURE 6, the data taken at 17.5° C. are plotted in this fashion and the straight line relationship predicted is obtained. In FIGURE 7, the same plot is presented for the observations at 24.5° C. The numerical analysis of these curves is incomplete at the present time and the lines drawn are provisional, but the following statements appear warranted.

The relation between total number of fissions completed and relative fission rate is different at 24.5° C. than it is at 17.5° C. Recovery to any given degree, say 90 per cent of normal, at 17° C. occurs when the cells have completed six fissions, but at 24° C. the same recovery is noted when eight fissions have been completed. In other words, the temperature rise has resulted in a change in the relationship between relative fission rate and the number of fissions. Dose apparently has nothing to do with this change except to determine where the recovery process begins. So these graphs indicate the recovery rate is separable from the number of fissions and is independent of dose. These are the relations predicted by the integral of the recovery function used to describe the recovery of fission rates.

At 32° C. (FIGURE 8), the relation is about the same as at 24° C. This does not necessarily indicate temperature independence in this region, but rather that the temperature dependence of restitution rate may be of the same magnitude as that of fission rate, to which it is being related. In the 17° to 24° C. range, the temperature dependence of the restitution constant (k) may be different from that of fission rate (ρ_s), resulting in the different values of the slope ($\rho_{s/k}$) observed at these two temperatures. Calculations are still pro-

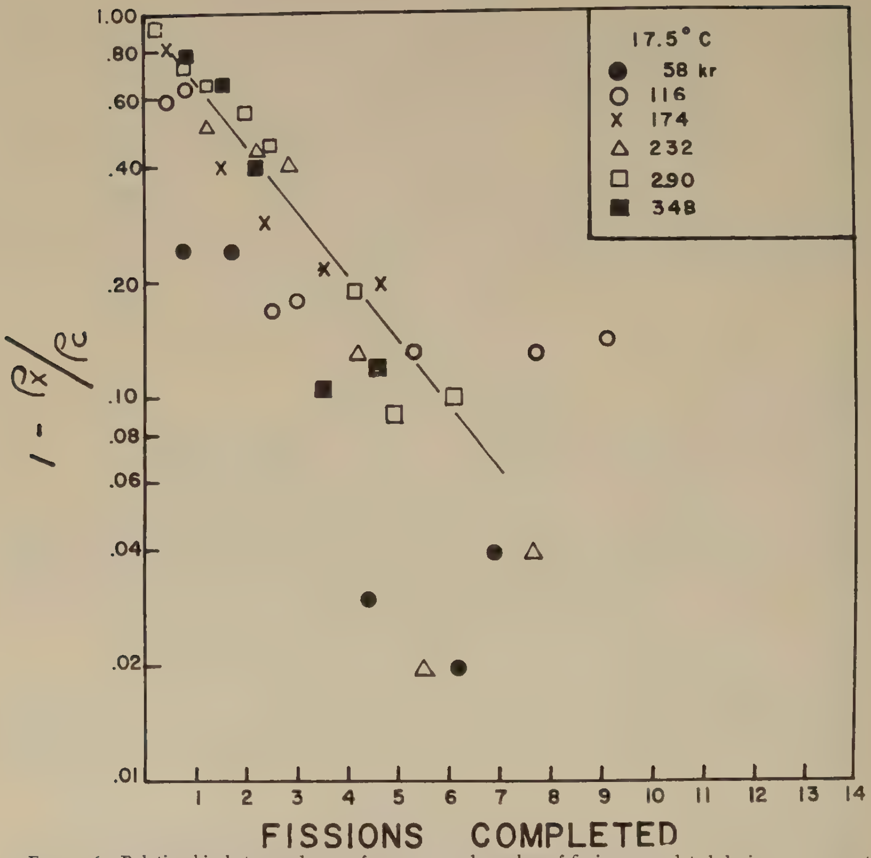

FIGURE 6. Relationship between degree of recovery and number of fissions completed during recovery at 17.5° C. The line drawn is a provisional estimate.

visional, and it is not yet possible to write firm values for these constants at all temperatures. Without them, however, discrimination between the fission process itself and the recovery of the determiners of fission is still possible from the graphs of the results at 17.5° and 24.5° C.

These preliminary results of the analysis say the following things concerning cell division rate and its recovery following X irradiation in *Paramecium*:

(1) Damage to the cell division mechanism is gradually recovered from over a series of cell divisions.

(2) The extent of damage, measured by the degree of reduction of fission rate, is a function of dose given (presumably, it also is a function of dose rate, and, conceivably, of linear energy transfer and other factors).

(3) The recovery of the cell is best described by a logistic function. This use presumes that normal division obtains when a minimum number of determiners of fission are present in the cell, that fewer than this number result in a division rate which is some fraction of normal, that the determiners are inacti-

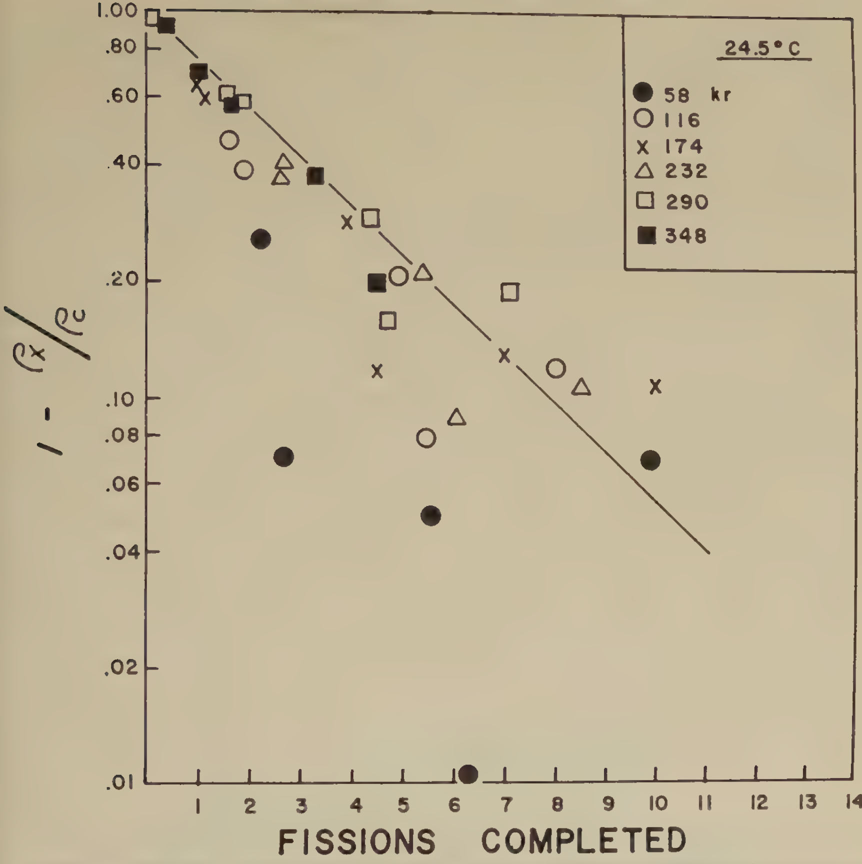

FIGURE 7. Relationship between degree of recovery and number of fissions completed during recovery at 24.5° C. The line is a provisional estimate.

vated exponentially by X irradiation, and that they are restored autocatalytically during the recovery process.

(4) The recovery rate is a function of temperature, being greater at high temperature.

(5) However, even though fission itself is more frequent at higher temperatures, recovery appears not to be causally related to the number of fissions, although it bears a particular relation to it at any given temperature.

(6) Recovery rate is also independent of the extent of the damage suffered (*i.e.*, of total dose given) within the dosage ranges tested.

Observation on gradual recovery of cell division processes after damage by X rays, UV, and HN2 have been reported by a number of authors: by Friedenwald and co-workers,[11, 12, 13] in corneal epithelium of mammals; by Giese,[17] in *Paramecium* and other forms; by Kimball,[23] in *Paramecium*; and by Gaulden *et al.*,[14] in grasshopper neuroblasts. There are numerous other investigations that are not treated here, and reference may be made to the following reviews

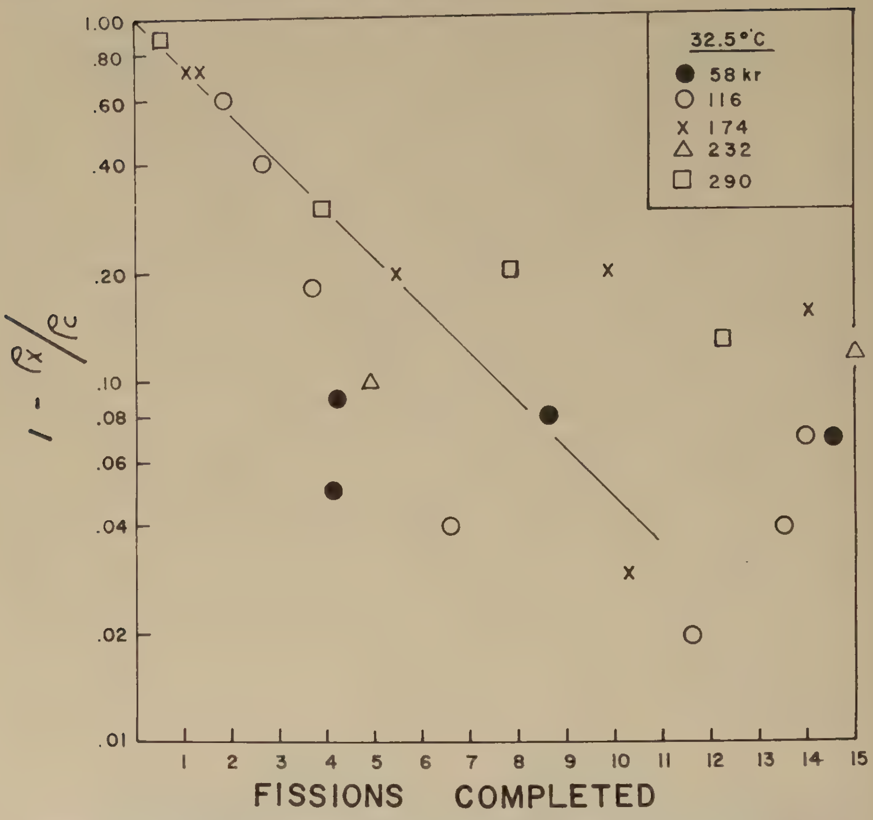

FIGURE 8. Relationship between degree of recovery and number of fissions completed during recovery at 32.5° C. The line drawn is provisional. Note that, in the region between 0 and 80 per cent of control fission rate, the predicted relationship between log $(1 - \rho_x/\rho_c)$ and number of fissions is obtained.

for description of them: Latarjet,[26] Patt,[29] Giese,[16, 17] Sparrow and Forro,[39] Gray,[19, 20] and the "Symposium" in the *British Journal of Radiology* for January and February, 1954.

There are instances in which suppression of cell division appears to be recovered from immediately. Ducoff, in *Tetrahymena*,[9] and *Chilomonas*,[10] working in mass culture, notes a cell division block after about 20,000 r. which lasts for approximately a cell generation, and from which the mass of cells recovers immediately if provided the proper nutrients. Since the recovery process is not spread over several generations, this phenomenon may be somewhat different from the one observed in *Paramecium* and, in one way, similar to that seen by Halberstaedter and Back[21] in *Pandorina*, another flagellate. In this organism, recovery, if it occurs, is complete by the first cell division in all survivors. In *Pandorina*, however, no delay of the time of cell division was seen, as was observed in *Chilomonas*.

The other observations on recovery appear to be consistent with those observed in the studies reviewed in this paper. Cook[8] observed no recovery of the fission mechanism in *Ascaris* eggs, when stored at 5° C., delay being approximately the same as that seen at 25° C. We might expect from the temperature dependence of the recovery phenomenon noted by us that, at very low temperatures, it would not proceed at all, and this result is consistent with ours.

Kimball *et al.*[23] observed gradual recovery of the fission mechanism over six to eight cell generations after X ray, HN2, and UV. X ray and HN2 are similar in causing greatest depression of fission rate immediately; while after UV maximal depression is observed in later posttreatment cell divisions, frequently the third and fourth.

The results reported by Kimball and his colleagues are not directly comparable to ours because of the different manner in which they are expressed, but superficial comparison indicates that recovery is proceeding in the manner described in this paper. Temperature effects on recovery were measured after ultraviolet treatment, the effect being that division time to the fourth division is decreased when temperature is decreased to 14° C. In other words, the effect of temperature after UV is opposite to that after X rays observed by us. These authors interpret the results in the following way: when the cell is irradiated, a substance (or substances) necessary for cell division and normally present in some excess is not produced at a rate necessary to maintain the level, and fission rate falls after a division or two. This fact explains the UV result, in which the long interval occurs some cell generations after irradiation.

Our notion, based on the temperature results after X ray, is different, in that the replacement rate of the substances is not affected by the total dose of X ray in these dosage ranges. Only the extent of the effect is dose-dependent, the recovery process being temperature but not dose-dependent. Of course, the absolute recovery observed at any one time depends on the amount of damage caused, which, in turn, is dose-dependent.

Friedenwald *et al.*[12] also postulated substances controlling cell division as a result of studies in the cornea—one that is immediately necessary for mitosis, and another that has to recover. In more recent work with X rays (Friedenwald and Sigelman[13]), a related idea is developed, *viz.*, that cell division appears to be dependent upon particulates, the number of which may be estimated from the S-shaped survival curves noted in their material. Their interpretation requires exponential manufacture of these substances, and a minimum amount present in a cell for mitosis. When a cell is injured, rate of recovery is independent of the damage incurred, and division proceeds when the particles are replaced to a threshold concentration. Our interpretation of the *Paramecium* data is similar in some respects, and is exactly the same in the important generalization that recovery (or replacement rate) is independent of the damage suffered. It is different in that division rates slower than normal must be accounted for, and this difference is allowed by letting the fission rate be proportional to the concentration of particles present. Some excess over the cell's immediate needs is produced and, in the steady-state condition, as much is used up at each cell division as is produced during that time interval at that temperature.

Reference to one other study in this connection can be made because it suggests another variable that may be of importance in the study of this phenomenon. Gaulden and his associates,[14] studying the effects of X rays on cell division of *Chortophaga* neuroblasts, contend that oxygen presence during irradiation does not affect the depth to which mitosis is depressed. Oxygen presence during X irradiation, however, as contrasted to treatment *in vacuo*, does affect some of the recovery processes: the time period of minimum mitotic activity is increased. The indication seems clear that, in grasshopper neuroblasts, the presence of O_2 during X irradiation affects recovery processes which, in *Paramecium*, we can now distinguish from the fission process itself.

Summary

(1) The effects of X rays in *Paramecium aurelia* with respect to three phenomena; namely, cell death, death after autogamy, and blockage of cell division, are described generally, and modifications of these effects by postirradiation temperature changes are presented and analyzed.

(2) At room temperature (24.5° C.), the LD_{50} dose for starved paramecia irradiated in salt solution is 200,000 r. When the temperature of the culture fluid, to which they are transferred after irradiation is 17.5° C., the LD_{50} dose is 260 kr., in general agreement with the observations of many others who find that survival of cells after X irradiation is higher at lower postirradiation temperatures.

(3) Interpretation of postautogamous death as a measure of lethal mutation induction leads to the conclusion that X-ray induction of lethals in *Paramecium*, as in most other organisms, is independent of postirradiation temperature.

(4) Suppression of fission by X rays is recovered from gradually over a series of cell divisions after they are resumed. At higher dosages greater reductions are observed.

(5) Recovery rate, however, appears to be dose-independent, although it is temperature-dependent, being higher at higher postirradiation temperatures.

(6) The logistic treatment of the responses at different temperatures indicates that a distinction between the replacement rate of cell division determiners and cell division itself is possible.

Acknowledgements

The technical assistance of Anne Bannon and Janet Fraembs in some of the experiments is acknowledged. Doctors C. F. Ehret and G. A. Sacher reviewed the manuscript, the latter participating in the development of the treatment contained in the section on suppression of fission.[32, 36]

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Discussion of the Paper

Doctor R. F. Kimball (*Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.*): I wish to point out that while Doctor Powers' treatment gives valuable information, especially for data from experiments with X rays, it is incomplete. Kimball *et al.** have shown by hourly observations that division delay in *P. aurelia* consists of two phenomena which can be partially separated experimentally. The first division is considerably delayed; the second is less affected; the third or fourth, or both, require longer times and are followed by a fairly rapid recovery of the normal rate requiring three to four divisions at the most. Thus, a distinction must be made between the effect on the first division and a late effect, with its maximum two to three divisions after irradiation. The late effect is very marked following ultraviolet irradiation, but is small relative to the delay in the first division following X irradiation. It is very difficult to detect in data from X-ray experiments when only daily observations are available. A comparison of Doctor Powers' data with ours leads me to believe that his method measures mainly the recovery from the late effect and is only slightly influenced by the original development of the injury. A comparison of one of our experiments with ultraviolet (*loc. cit.*) with his results supports this view. We found that low temperature, during the first 24 hours after irradiation, decreased the late effect but had little if any influence on the delay in the first division. This effect leads to an increased percentage recovery for a given division after irradiation and so compared rather well with Doctor Powers' X-ray data. I believe that Doctor Powers' work furnishes a very valuable technique for quantitative analysis of the recovery from division delay by X rays. The theoretical implications of the formulae, however, must be considered as first approximations only, though valuable ones; and the method would not be applicable without considerable modification to cases, such as are found with ultraviolet, in which the separation between the early and late effects is more pronounced.

Doctor E. L. Powers: The difference in procedure, in the two instances, should be re-emphasized. Before exposure of the cells to irradiation, we allowed them to remain in salt solution overnight in order to insure in the group of cells as great a degree of homogeneity as possible. Kimball *et al.* attempted to produce homogeneity by choosing cells which had divided no earlier than two hours before irradiation. We irradiated starved animals in salt solution. The other authors used well-fed animals in organic culture fluid. Perhaps the metabolically different states in which the animals are at the time of and immediately following irradiation may explain the presence of the so-called "early" effect in Kimball's experiments and of its absence from ours.

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* Kimball, R. F., R. P. Geckler & N. Gaither. 1952. Division delay by Radiation and Nitrogen mustard in *Paramecium*. J. Cellular Comp. Physiol. **40:** 427–459.

Whether this is true or not, however, its presence in the Kimball data (derived from well-fed animals); its lack of temperature dependence in the temperature range tested; its absence from our data (derived from starved animals); and the demonstrated relation between metabolic constituents and growth rate of other irradiated microorganisms (Ducoff*) make the early effect a phenomenon of considerable interest. But the description and general analysis of recovery presented in this paper is not expected to explain all departures from the smooth recovery that is indicated by the organisms used in these experiments.

* Ducoff, H. S. 1953. Factors influencing the duration of postirradiation cell division block in *Chilomonas paramecium*. Dissertation. Univ. of Chicago. Chicago, Ill.

THE ROLE OF OXYGEN AND PEROXIDE IN THE PRODUCTION OF RADIATION DAMAGE IN *PARAMECIUM**

By R. F. Kimball

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It has been known, for some time, that damage to chromosomes by X rays is less when the irradiation is carried out in the absence of oxygen. Giles[1] reviewed the earlier work on this problem and gave a number of reasons for believing that oxygen acts by favoring the formation of hydrogen peroxide which, in turn, breaks the chromosomes. Recent work by Schwartz,[2] Baker and Von Halle,[3] and Swanson and Schwartz[4] makes it plain that oxygen must influence the rejoining processes whether it also influences breakage or not. Wolff and Atwood[5] have presented evidence that both the time over which ends remain open and original breakage are affected. Thus, the hypothesis advanced by Giles[1] is certainly incomplete in that it recognizes only an effect on breakage.

However, the theory that oxygen exerts its influence by reactions involving the immediate products of radiation seems quite likely, in several cases,[6] and recently, considerable information about this matter has been reported by several investigators who used bacteriophage, DNA, or proteins. This paper presents evidence that H_2O_2 is not involved in an important way in the production of mutations and compares the evidence from cytogenetic studies and investigations on bacteriophage, DNA, and proteins in an attempt to achieve a more complete picture of the nature of oxygen action. The experimental work was done with the ciliate protozoan, *Paramecium aurelia.*

Experimental Methods

The methods for measuring mutation in *P. aurelia* have been given by Kimball.[7] Briefly, treated paramecia are isolated, each into a separate container, allowed to multiply vegetatively, and then sent through the self-fertilization process of autogamy. From each original treated animal, 25 autogamous animals are isolated, and the 25 clones produced from them are checked for survival and amount of growth. The percentage of these 25 clones which grow at normal rate is the measure used, while the arc sine transformation is used in statistical tests of significance.

The absolute value of the natural logarithm of the average fraction of normal clones increases nearly linearly with a dose of X rays and, on the simplest theory,[7] is approximately equal to half the average number of mutations per micronucleus. The two diploid micronuclei of *P. aurelia* contain from 60 to 80 chromosomes, and the group of 25 autogamous clones tests for mutations in essentially all of these. Thus 60 treated paramecia furnish a test for mutations from 3600 to 4800 chromosomes. However, the method is subject to a large variance, so that a difference between two groups of 60, of about 0.5 mutations per micronucleus, is significant at the 5 per cent level, when the

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* This work was performed under Contract No. W-7405-Eng-26 for the United States Atomic Energy Commission.

TABLE 1

EFFECT OF OXYGEN PRESENT DURING BUT NOT BEFORE AND AFTER IRRADIATION

Gas present			N*	P*	M*
20 minutes before irradiation	During 2-minute irradiation	20 minutes after irradiation			
20% O_2	20% O_2	20% O_2	80	70.5	0.70
N_2	20% O_2	20% O_2	40	68.5	0.76
N_2	N_2	20% O_2	40	86.5	0.29

* In this and subsequent tables, N is the number of original treated paramecia from which groups of 25 autogamous animals were obtained. P is the percentage of these 25 N autogamous animals which were normal. M equals $-2 \ln (P/100)$ and is approximately equal to the number of mutations per micronucleus.

average number of mutations is 1.4 per micronucleus (50 per cent of clones normal). When the average number is 0.18, a difference of about 0.15 can be detected.

The exact nature of the mutations has not been determined. Kimball[7] gives reasons for believing that mainly small changes, such as small deletions and gene mutations, are involved. More extensive two-break rearrangements, however, may contribute appreciably at higher doses.

Further Information on the Oxygen Effect on Mutation in Paramecium

Kimball and Gaither[8] have shown that more mutations are produced when the paramecia are irradiated in air or oxygen than when irradiated in nitrogen or helium. It has now been shown that this effect is dependent on oxygen present during, but not before or after, irradiation (TABLE 1). The amount of mutation in paramecia which had been in nitrogen just before irradiation, but had oxygen present during irradiation, was not different from the amount in those which had been in oxygen all along. Paramecia which were in nitrogen through irradiation but were put into oxygen immediately afterwards showed as much decrease in mutation as is found when oxygen is withheld not only during irradiation, but for some time afterward. The paramecia were in small hanging drops over a small cavity, so gas exchange probably required only a few seconds. About a half minute elapsed between gas exchange and the beginning or end of the X-ray exposure.

Tests for an Effect of H_2O_2 on Mutation Production

Despite this evidence that oxygen acts at the time of irradiation and so, presumably, in the chemical reactions immediately following the absorption of the energy, Kimball and Gaither[9] found no mutagenic effect of H_2O_2 in the medium surrounding the cells. At the time, it seemed possible that insufficient penetration of the peroxide was the cause of the negative findings. Recent calculations by J. Z. Hearon of the Mathematics Panel of Oak Ridge National Laboratory, however, based on diffusion theory and experimentally determined constants for catalase activity and permeability to H_2O_2 of *Paramecium aurelia*, have indicated that about 1 per cent of the outside concentration probably is present in the nuclei. The calculations and data on which they are based will be published elsewhere. The results suggest that the concentrations of peroxide inside the nucleus in some of the negative experiments were probably not much less than those produced in pure aerated water by 3000 r. of X rays (about 5×10^{-6} M from Savage's data[10]). Barron[11] has pointed out that still less H_2O_2 would be formed in biological material, and so the concentration produced within the nuclei by 3000 r. is probably less than 5×10^{-6} M. A dose of 3000 r. is sufficient to reduce the percentage of normal clones, after autogamy, to about 50 per cent, when the irradiation is in air, and to about 76 per cent, when it is in nitrogen. Therefore, if most of the oxygen effect were due to H_2O_2, there should have been a detectable effect of H_2O_2 in the experiments by Kimball and Gaither.[9]

However, the uncertainties of the calculations of penetration make this conclusion relatively unsafe, and so other tests were made by increasing the average concentration of H_2O_2 in the cell by poisoning catalase with 0.003 M KCN. The paramecia withstood this concentration of cyanide well, as long as the concentration of H_2O_2 used with it was not too high. Measurements of the destruction of H_2O_2 by living paramecia suggest that the concentration of H_2O_2 inside the nucleus was probably 50 per cent of the outside concentration when cyanide was used. Even under these circumstances, no effect of H_2O_2 was found as shown in TABLE 2. The concentration inside the cells in this experiment was probably 30 times as high as that which would be produced in pure aerated water by 3000 r.

Despite this negative finding, the conclusion that H_2O_2 is not effective requires several other tests. Alper[12] has shown that irradiated bacteriophage are considerably more sensitive to H_2O_2 than are unirradiated ones. To test the possibility that the same thing is true for mutations, paramecia were exposed to peroxide for four minutes before, two minutes during, and four minutes after irradiation with 3000 r. TABLE 3 shows that no appreciable effect of the added H_2O_2 was found. Very surprisingly, however, it was found that a 10-minute exposure to H_2O_2 after irradiation decreases the detectable mutation (TABLE 4). The explanation is not clear, but this finding makes it certain that H_2O_2 remaining in the cell and acting for a time could not account for the oxygen effect.

These experiments still leave the possibility that the high concentrations of H_2O_2, which would be found momentarily along the tails of electron tracks, could be responsible for the effect. The concentration present in electron

TABLE 2

LACK OF AN EFFECT OF H_2O_2 ON CYANIDE-POISONED PARAMECIA

Fluid used for a 10-minute treatment	N	P	M
Ringer's solution	60	93	0.15
2.4×10^{-4} M H_2O_2	60	96	0.08
3.5×10^{-4} M H_2O_2	51	96	0.08
0.003 M KCN	56	99	0.02
0.003 M KCN & 2.4×10^{-4} M H_2O_2	58	96	0.08
0.003 M KCN & 3.5×10^{-4} M H_2O_2	47	98	0.04

TABLE 3

LACK OF AN EFFECT OF RADIATION-PRODUCED PEROXIDE PRESENT DURING IRRADIATION

X-ray dose (r).	Fluid present during irradiation	N	P	M
None	Ringer's	30	96.4	0.07
3380	0.003 M KCN	42	71.3	0.68
3380	0.003 M KCN & 1.8 × 10^{-4} M H_2O_2*	52	66.8	0.81
None	Ringer's	24	97.8	0.04
3390	0.003 M KCN	71	42.5	1.71
3390	0.003 M KCN & 1.3 × 10^{-4} M H_2O_2*	70	47.5	1.49

* H_2O_2 produced by X-irradiating Ringer's solution and determing the concentration colorimetrically.

TABLE 4

EFFECTS OF POSTTREATMENT WITH H_2O_2 ON DETECTABLE MUTATIONS INDUCED BY X RAYS

X-ray dose (r.)	Posttreatment	N	P	M
None	None	20	98.6	0.03
2860	None	60	53.8	1.24
2860	1.2 × 10^{-4} M H_2O_2	36	72.9	0.63
None	None	20	98.4	0.03
2800	None	60	50.7	1.36
2800	0.7 × 10^{-4} M H_2O_2	60	65.1	0.86

tracks cannot be calculated with any certainty since there seems to be considerable disagreement about the events immediately following ionization in liquid water (see, for example, Dewhurst, Samuel, and Magee[13]). Gray[6] has estimated that the concentration of H_2O_2 in alpha particle tracks may be as high as 1 M. He also points out, however, that oxygen plays an important role in producing peroxide only in tracks with considerably less dense ionization, as in those produced by X rays. Thus, it is probable that only concentrations appreciably less than 1 M need be considered. It has proved possible to expose paramecia for 30 seconds to concentrations of 0.2 to 0.15 M commercial peroxide in the presence of 0.003 M KCN. TABLE 5 shows no mutagenic effect of this treatment. It can be calculated that the time to reach the final equilibrium concentration inside the cell is a few seconds, so that the nuclei were exposed to high concentrations for an appreciably longer period than the duration of localized high concentrations in electron tracks.

Tests for mutation could be made, of course, only with paramecia which survived the harmful immediate effects of the exposure to H_2O_2 and KCN. In some experiments, many animals died before the first division following the treatment, so that selection must be considered. Kimball[14] has already reviewed the reasons for considering such death before the first division as nongenetic. In particular, it seems extremely improbable that mutations in the diploid micronuclei would have any appreciable effect on vegetatively multiplying animals, since the highly polyploid macronucleus should contain

TABLE 5

LACK OF A MUTAGENIC EFFECT OF HIGH CONCENTRATIONS OF H_2O_2

Experiment	Fluid	N	P	M
1	0.003 M KCN	14	88.0	0.26
	0.003 M KCN & 0.2 M H_2O_2	6	85.3	0.34
2	0.003 M KCN	72	89.0	0.23
	0.003 M KCN & 0.15 M H_2O_2	27	87.5	0.27
3	0.003 M KCN	87	96.9	0.06
	0.003 M KCN & 0.15 M H_2O_2	17	98.1	0.04
4	0.003 M KCN	51	95.8	0.09
	0.003 M KCN & 0.15 M H_2O_2	17	95.5	0.09

many unmutated chromosomes homologous with those which are mutant in the micronucleus. The possibility remains that selection favored animals in which the peroxide concentration was low. The animals used for treatment were all in about the same stage of the division cycle, and so were of approximately the same size. Although variations in internal concentration might cause minor variation in response, it seems most improbable that they could entirely prevent the detection of a mutagenic effect.

One further hypothesis on which there is some suggestive negative evidence in another organism is that organic peroxides, rather than H_2O_2, are the main mutagenic agent. This hypothesis has been advanced by several workers (*e.g.*, Wagner *et al.*[15]). Dickey, Cleland, and Lotz[16] give a comparison between the mutagenic effects of several agents, including organic peroxides, H_2O_2, and X rays on *Neurospora*. Their data show that organic peroxides are more effective than H_2O_2. These investigators found that a 30-minute exposure to 0.09 M hydroxymethyl *tert*butyl peroxide (the most effective peroxide tried) produced approximately the same amount of mutation as 30,000 r. of X rays. If every ionization produced by this dose of X rays gave rise to a molecule of organic peroxide, it can be calculated that the concentration would be approximately 10^{-4} M. Therefore, only the most extreme assumptions about the permeability of *Neurospora* spores to the peroxide could make these results consistent with the hypothesis that an appreciable portion of the total mutations produced by X rays is produced by diffusible organic peroxides.

The conclusion that hydrogen peroxide (and probably diffusible organic peroxides) is not important in the production of mutations by X rays still leaves many possibilities for interpreting the oxygen effect in terms of radiation chemistry. For example, as has been repeatedly pointed out, the HO_2 radical could act as a mutagen. Daniels, Scholes, and Weiss[17] have suggested that oxygen could interfere with the back reaction of OH with H to form water by competing for H atoms and thus making more OH radicals available for other reactions, and they also point out that the same competition might occur between oxygen and organic radicals. Presumably, this latter competition could occur for radicals produced *in situ* in the chromosome, thus making less likely the back reaction with H to restore the original condition. Thus it is not necessary to invoke the diffusible mutagen hypothesis for the oxygen effect, even though it is a possibility. No doubt, a wide variety of such hypotheses could be made. The point to be emphasized here is that the idea that a relatively stable chemical mutagen is involved finds no support in those experiments in which quantitative comparisons have been made between the action of certain of the presumed mutagens and X rays.

Comparisons with Studies on Bacteriophage, DNA, and Pepsin

Direct tests of some of the other possible hypotheses of the chemical action of oxygen on the chromosomes are difficult if not impossible to make at present. However, direct tests are possible on bacteriophage, DNA, and proteins. The recent experiments with bacteriophage summarized by Alper[12] suggest that the only decrease in effect due to decreased oxygen tension involves the so-called "aftereffect." Inactivation of phage continues for some time after the exposure to X rays has ceased. By a series of ingenious experiments, Alper[12] has been able to show that this effect can be accounted for by the action of H_2O_2 acting on phage previously injured by radiation, and the amount of H_2O_2 formed is oxygen-dependent. The original injury, which is brought to light by peroxide treatment, is apparently due to reducing radicals rather than oxidizing ones. Alper believes that reducing radicals also brings about the immediately detectable "indirect" inactivation of the phage. She shows that decreased oxygen tension increases these effects slightly.

There is some disagreement about the details of the inactivation of DNA by X rays as brought out by the discussions of Scholes and Weiss, of Butler, and of Conway, following the paper presented by Conway[18] at a recent symposium on aftereffects. Apparently, there is little effect of oxygen on the immediately detectable changes in the nucleic acid, or the effects may even be increased by decreased oxygen tension. However, there is an oxygen-sensitive aftereffect. Conway[18] suggests that this effect is, in part, the result of H_2O_2 acting after irradiation in a manner very similar to its action on bacteriophage[12] and, in part, to the formation of unstable peroxide groups in the DNA molecule by oxidizing radicals, whose formation, in turn, is influenced by oxygen. Scholes and Weiss[19] find appreciable, though reduced, aftereffect in the absence of oxygen, and they suggest that the aftereffect is due to labile phosphate esters formed by oxidizing radicals.

Anderson[20] finds that there is little if any effect of oxygen on the immediately detectable inactivation of pepsin, but that the slow inactivation which continues for many hours is oxygen-dependent. He presents convincing evidence from dilution and dialysis experiments in support of the assertion that the injury which is slowly expressed is not due to the action of some substance such as peroxide, but to instabilities in the pepsin itself.

These results are in remarkable agreement in showing that the oxygen-sensitive portion of the effect develops slowly, whereas the immediately detectable effects seem to be oxygen-independent or even to be slightly greater in the absence of oxygen. However, two entirely different mechanisms have been proposed: the peroxide hypothesis of Alper,[12] and the hypothesis of unstable states induced by oxidizing radicals advocated by Conway[18] and by Scholes and Weiss.[19] The data given in this paper exclude the peroxide hypothesis for mutations in *Paramecium* and, by a reasonable extension, for mutations and chromosome aberrations in other organisms. This hypothesis is also improbable on *a priori* considerations, since it seems unlikely that peroxide could persist long enough in most cells to bring about an appreciable amount of what appears from the data on phage to be a rather slow reaction. The exclusion of the peroxide hypothesis does not require acceptance of the second hypothesis for chromosomal changes, but it does make it necessary to consider the latter hypothesis seriously.

The investigations of Schwartz,[2] Baker and Von Halle,[3] and Swanson and Schwartz[4] have shown that oxygen is concerned in reunion processes, while Wolff and Atwood[5] have shown that both the time over which breaks remain open and original breakage is modified. These investigations, however, do not show precisely how the modification of reunion and of detectable breakage is brought about. The *in vitro* studies are suggestive in showing that not all effects of X rays on nucleic acids and proteins are equally modified by oxygen, and that the time course of the appearance of the different types of damage is different. It may be hoped that the data on the oxygen effect will, in time, help to make possible precise identification of the changes leading to genetic damage with the *in vitro* changes in nucleic acid and protein. More information about both the *in vitro* and the cytogenetic effects, however, will be required before such identifications can be considered as more than guesses.

Other Effects of X Rays on Paramecium

Studies of several other types of radiation damage with *Paramecium* have yielded information which it is of interest to compare with the mutation data and with the results on phage, nucleic acid, and protein. Kimball and Gaither[8, 9] have shown that division delay by high doses (20 to 300 kr.) of X rays can be due to a large extent to H_2O_2 when this substance is allowed to accumulate in the medium surrounding the cells. When peroxide is prevented from accumulating, however, oxygen has no effect. Kimball and Gaiter suggest that peroxide produced locally within the cell is unable to reach sufficient concentration or persist long enough to be effective. This suggestion means that radiation absorbed locally within the cell acts through an oxygen-independent pathway. The situation resembles that in bacteriophage,[12] in that the effects produced when H_2O_2 is eliminated are nearly oxygen-independent. In fact, there is an indication, in some experiments with *Paramecium*, of a greater effect in reduced oxygen concentration which makes the situation still more like that for bacteriophage. The case differs in that no evidence has been found showing that paramecia are sensitized to peroxide by X rays.

High doses (10 to 50 kr.) of X rays also cause disturbance of the behavior of the gamete nuclei at conjugation.[21] FIGURE 1 shows that oxygen has no effect on the inhibition of pronuclear exchange. It is possible that dominant lethal changes are involved in this effect. If so, the results are similar to those of Baker and Von Halle[3] for *Drosophila*, and of Russell, Kile, and Russell[22] for mice, since these workers also found, at most, a small effect of oxygen on dominant lethals. Baker and Von Halle's explanation[3] might be applicable here.

Two closely related DNA-protein particles are found in certain stocks of *P. aurelia*, though not in the one used for the mutation work. These particles have been called kappa and paramecin. Kappa is a self-reproducing particle in the cytoplasm of the paramecia, and is detected either by cytological examination or by its ability to multiply. Paramecin is secreted into the medium by stocks containing kappa. It is usually detected in breis of paramecia by its ability to kill sensitive paramecia. Extensive studies on the X-ray inactivation of these two particles have been published by Nanney.[23]

Geckler and Kimball[24] have shown that kappa is more readily inactivated by X rays when oxygen is present during irradiation than when it is absent. Oxygen present before or after irradiation was ineffective. They also found no effect of H_2O_2 in the medium. Cyanide poisoning was not used, but fairly

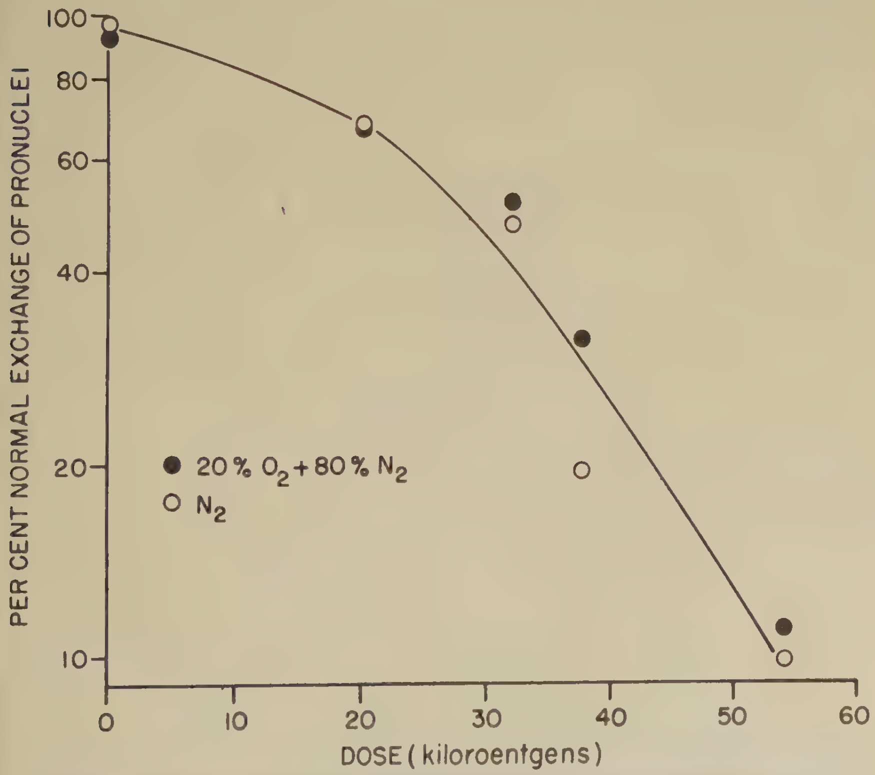

FIGURE 1. Percentage of conjugant pairs showing normal exchange of pronuclei plotted against dose in kiloroentgens. The individual points are averages of several experiments.

high (1×10^{-3} M) concentrations of H_2O_2 proved usable, so that it seems probable that peroxide reached the particles in concentrations of about the same magnitude as would be produced by X rays.

A few experiments have also been done in the X-ray inactivation of paramecin in breis of broken-up paramecia. Since the technique was not completely perfected, the difference between the form of the curves in FIGURES 2 and those of Nanney[23] may well be due to technical difficulties. Despite this, FIGURE 2 shows such a clear-cut oxygen effect in the opposite direction from normal that it seems worth while to record it. The curves resemble, in some respects, Alper's curves[12] for the immediate indirect effect on bacteriophage. It is possible to bring this case entirely in line with Alper's work[12] with phage, if it is assumed that the brei was sufficiently dilute to make action by reducing radicals possible while an aftereffect by peroxide was prevented by a high catalase content. The difference between the results with kappa and paramecin are understandable if it is remembered that different properties of the particles are tested, and so different kinds of damage may have been involved.

Summary

It can be shown that the effect of reduced oxygen tension in lowering the X ray-induced mutation rate in *Paramecium aurelia* cannot be attributed to a decreased production of H_2O_2, since this substance is ineffective as a mutagen

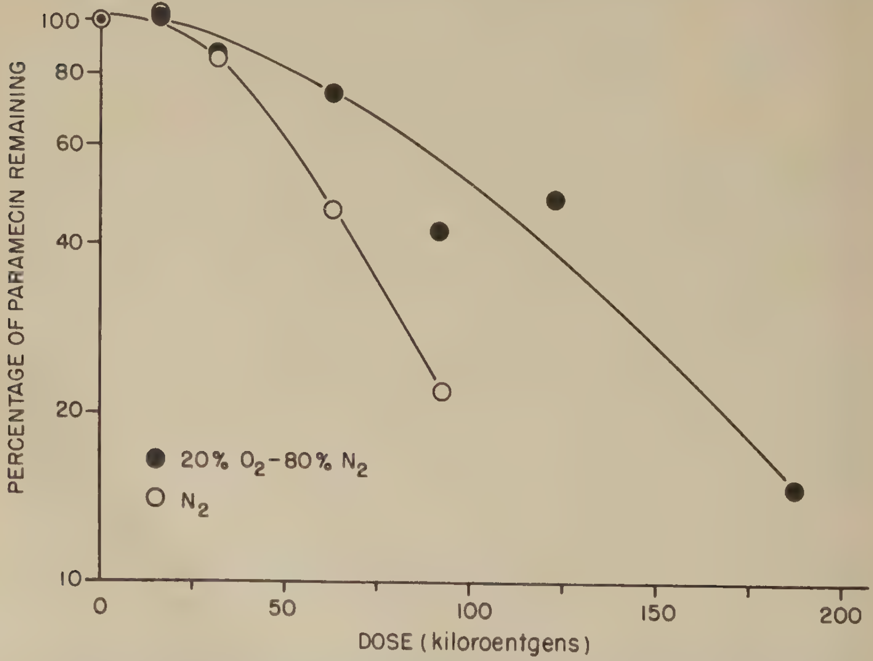

FIGURE 2. The survival of paramecin particles expressed as a percentage of the particles in the control plotted against dose in kiloroentgens. Each point is the average of two or more points from different experiments.

either by itself or in combination with X rays. This effect could mean that the oxygen-dependent part of the mutagenic effect is brought about by short-lived radicals acting as mutagens; but it is emphasized that a diffusible chemical mutagen interpretation is by no means required even if, as seems probable, oxygen takes part in the chemical reactions immediately following the absorption of the energy of the radiation. Comparison of the role of oxygen in the production of various kinds of radiation damage to *Paramecium* and a brief review of the work of others on bacteriophage, DNA, and protein strongly suggests that oxygen may play a variety of roles, and that there are no real grounds for expecting uniformity in different biological materials.

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Discussion of the Paper

Doctor E. L. Powers (*Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Ill.*): Please comment on the validity of using postautogamous effects as a measure of damage inflicted upon microneuclei by treatment.

Doctor Kimball: Several reasons for believing that the postautogamous effects of X rays are the result of genetic damage to the micronuclei are given by Kimball (Genetics. **34:** 412, 1949). Exceptional cases which do not fit this hypothesis have been found both in published and unpublished work. However, most unpublished work, to the best of my knowledge, has continued to support this hypothesis, and the exceptional cases have not been sufficiently analyzed to ensure that a somewhat modified form of the mutation hypothesis could not account for them. Thus most of the postautogamous effects after X-ray treatment can be accounted for by the mutation hypothesis, and the exceptional cases have not been clearly accounted for by some other hypothesis.

Since this paper was submitted, it has been shown that the relation of M to X-ray dose departs significantly from linearity. This finding does not alter the conclusion just stated, but it does mean that the number of mutations per micronucleus may not be properly estimated by M.

FACTORS IN THE RADIOSENSITIVITY OF MAMMALIAN CELLS

By Harvey M. Patt

Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Ill.

The selective action of ionizing radiations was recognized very early in the history of their application to biological investigation. It was learned that radiosensitivity is frequently associated with proliferative activity and that the most dramatic effects of the radiations are related to their influence on growing or developing systems. Since the initial energy transfer is chemically nonspecific, sensitivity must obviously depend upon differences in the subsequent mechanisms of energy dissipation, or on the constitutional and physiological requirements of the responding cell or tissue. It is the purpose of this paper to discuss the influence of environmental factors on the response of mammalian cells to ionizing radiations. Some attention will also be given to the general problem of radiosensitivity by way of introduction. This area has been reviewed recently.[1-3] Although the expression radiosensitivity has attained wide usage, its meaning is ambiguous unless it is defined in terms of a standard dose response for a specific biological effect. Yet, even with this qualification, it is often a matter of conjecture as to whether sensitivity implies differences in the extent of the initial injury or in the capacity for recovery. In general, radiosensitivity, as discussed here, will refer to the destruction or degeneration of cells as living entities.

It is generally agreed that the proliferative capacity of a tissue is an important factor in its radiosensitivity. A relationship of this sort is understandable, since the cell in mitosis is usually more susceptible to injury than the cell at rest, and degenerative changes are prone to occur at the time of cell division regardless of the period of irradiation. Tissue sensitivity to X rays may be expressed as an inverse function of the intermitotic time in certain instances.[4] It may be recalled that the duration of mitotic inhibition in various tissues bears a direct relationship to the duration of interphase.[5, 6] Indeed, this relationship may give rise to the apparent paradox that tissues believed to be radiosensitive by histologic criteria may show less interference of mitotic activity than somewhat more resistant tissues.[6] The nature of the relationship between sensitivity and cell division is obscure. Peaks of respiratory and synthetic activity occur during the mitotic cycle, and one may inquire to what extent these and other changes, for example in chromosomal surface and cross section and in local oxygen tension, contribute to the sensitivity of premitotic and mitotic cells.[1]

It must be emphasized that there are many departures from the simple condition relating radiosensitivity to growth and differentiation. Perhaps the most glaring example, about which more will be said later, is the fact that small lymphocytes are quite sensitive and yet largely nonmitotic. We may note also that rapidly growing anaplastic tumors may be either more or less resistant than slowly growing neoplasms. There are other impressive exceptions to the general thesis that proliferation and primitiveness are common denominators for differences in radiosensitivity. These exceptions have been pointed out by Bloom,[7] Mole,[8] and others. It is sufficient to note for our purpose that mammalian cells may be injured and killed by moderate gamma or X irradiation through mechanisms apparently unrelated to an aberration in cell division or in growth generally. It may also be stated that sensitivity dependencies of this sort are, in general, less obvious with the high ion density radiations.

Brues[9] has suggested that radiosensitivity may be related to the ratio of the nuclear and cytoplasmic nucleic acids, since the highest ratio is found in lymphoid tissue and the lowest in resistant cells. Proliferating tissues probably show, in general, an increase in DNA per cell.[10] It is well known, however, that PNA also tends to be increased in proliferating cells. There is no obvious relationship between susceptibility of different tissues and their metabolic rate, nor is there any reason to believe that differences in oxygen tension of the various tissues are of a sufficient magnitude to influence sensitivity to X rays appreciably, although the liver may be a possible exception. On the other hand, tissue oxygen concentration is probably an important consideration in the radiosensitivity of poorly vascularized tumors, as may be inferred from the work of Gray[11] and Hall.[12] Although polyploidy may protect simple organisms against radiation damage,[13, 14] this characteristic would not seem to be an important factor in radiosensitivity of the several animal tissues. It may be applicable, however, to certain instances of tumor sensitivity.

There is growing awareness that radiosensitivity can be influenced, to a considerable extent, by the physiological interplay in the system as a whole, whether this represents a cell, tissue, or animal. It may be noted, for example, that X irradiation of isolated nuclei results in negligible change in nuclear structure[15] and in the properties of nucleoproteins.[16] There accordingly appears to be a metabolic requirement even for certain primarily local radiation effects.

Radiosensitivity of Lymphoid Tissues in Vivo *and* in Vitro

We may turn now to a more detailed consideration of the role of environmental factors in the radiosensitivity of mammalian cells. For this purpose, we shall draw mainly on experiences with lymphoid tissues. Although the lethal dose of X radiation varies among the different animal species, it is of interest that lymphopenia and lymphoid involution are more nearly independent of species, and that they reflect the amount of radiation instead of the lethal effect.[7, 17, 18] The great sensitivity of the lymph-node lymphocyte and of the thymus lymphocyte may be recalled. The fact that these cells rarely divide, and yet are as sensitive as mitotic cells has prompted Schrek and Ott[19] to suggest that a common physiological process may be affected in the nucleus of the lymphocyte and in the mitotic nucleus in general. This process is considered by many investigators to involve an aberration in nucleoprotein metabolism. Klein and Forssberg[20] have shown recently that there is a decrease in DNA per cell with time during the mitotic block of irradiated ascites tumor cells. The average cell volume, PNA and nitrogen contents increase, however, in proportion to the estimated growth rate before irradiation. A decrease in

TABLE 1

COMPARATIVE EFFECTS OF X IRRADIATION ON LYMPHOID CELLS

Cell or tissue	Effect*	Time after X irradiation	ED_{50}† roentgens	Regression (log-arc sine)	Reference
		In vitro			
Lymph-node lymphocytes	Pyknosis	5 hours	305 ± 6	0.99 ± 0.02	21
Blood lymphocytes	Pyknosis	5 hours	2218 ± 780	0.59 ± 0.07	21
Thymus lymphocytes	Eosin stainability	6 hours	518 ± 10	0.94 ± 0.08	27
Thymus lymphocytes	Eosin stainability	22 hours	114 ± 13	1.56 ± 0.12	24
		In vivo			
Lymph-node lymphocytes	Pyknosis	5 hours	160 ± 4	1.67 ± 0.05	21
Duodenal lymphocytes	Pyknosis	5 hours	2805 ± 499	1.02 ± 0.09	21
Blood lymphocytes	Lymphopenia	3 days	137 ± 8	1.84 ± 0.12	26
Thymus	Involution	3 days	180 ± 16	1.49 ± 0.19	26
Thymus	Involution	5 days	194 ± 6	1.34 ± 0.04	25
Spleen	Involution	3 days	270 ± 19	1.14 ± 0.09	26
Spleen	Involution	5 days	242 ± 15	1.05 ± 0.07	25

* Expressed as percentage of nonirradiated.
† Dose required for 50% effect.

the DNA/PNA ratio per cell has also been observed in the X-irradiated thymus.[20A]

Trowell[21] has contributed a significant paper on the radiosensitivity of lymphocytes in which he emphasizes the nonspecific nature of the cytological damage produced by X rays. The dose required to produce nuclear pyknosis in 50 per cent of rat-lymph-node lymphocytes five hours after irradiation (ED_{50}) is about 300 r. in tissue culture and 160 r. *in vivo.** This difference may be obscured somewhat by differences in oxygen concentration and in metabolism in the two situations. The results, however, compare favorably with the observations by Schrek[22, 23] and Patt *et al.*[24] on the *in vitro* sensitivity of rabbit thymus lymphocytes as estimated by eosin stainability and with the 50 per cent dose for lymphopenia and thymus and spleen involution, after whole body X irradiation of the mouse, as shown by Carter[25] and Patt.[26, 27] The data are summarized in TABLE 1.

These effects are reasonably linear with the log of the dose as indicated in FIGURES 1 and 2.† The linearity of these relationships is suggestive of a symmetrical distribution of sensitivities. Variations in slope reflect differences in the range of sensitivities. It may be noted, in particular, that the slope for the lymph node lymphocyte response is steeper *in vivo* than *in vitro*. This effect is probably not a result of differences in the mean oxygen concentration, since the slopes do not vary with oxygen tension.[28] There is no difference,

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* The data reported by Trowell[21] were corrected for pyknosis in the nonirradiated samples.

† An arc-sine transformation was used; essentially similar relationships appear with a probit transformation. The regression coefficients were kindly determined by Sylvanus Tyler.

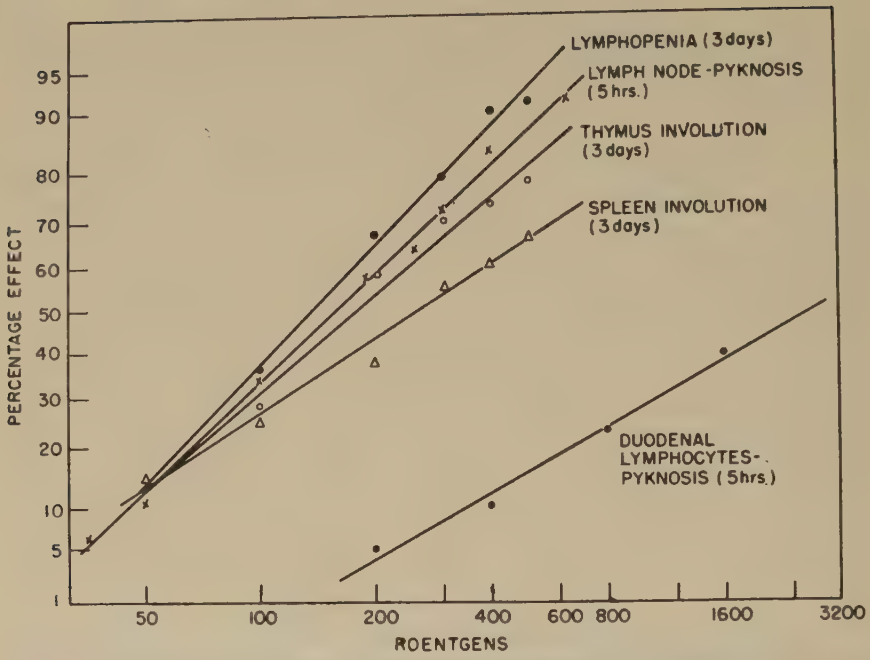

FIGURE 1. Comparative *in vivo* effects of X irradiation on lymphoid cells. (Lymph node and duodenal lymphocyte data from Trowell[21]; blood lymphocyte and thymus and spleen data from Patt *et al.*[26, 27])

moreover, between the slopes for thymus lymphocyte suspensions, in which the oxygen gradient is comparatively small, and for lymph node lymphocyte cultures at comparable times (FIGURE 2). The apparent difference in range of sensitivities for the lymph node may reflect differences in the reaction time *in vivo* and *in vitro.* It is noteworthy that the slope for thymocytes is 0.94 at six hours after X irradiation and 1.57 at 22 hours. The latter may be compared with a slope of 1.67 for lymph node pyknosis at five hours *in vivo.*

The wider range of sensitivity for blood lymphocytes than for lymph node or thymus lymphocytes at comparable times *in vitro* may be attributed to the fact that these cells represent a more heterogeneous population in terms of maturity and differentiation. Trowell[21] has suggested that this may also account for their lower average sensitivity. Lymphocytes of the duodenal villi appear to be much more resistant to X rays than lymph node lymphocytes *in vivo.* The relative resistance of scattered intestinal lymphocytes may be related perhaps to their age or to environmental factors, *e.g.*, oxygen. It is possible also that pyknotic lymphocytes are removed more rapidly from the intestinal mucosa than from a lymph node, and this difference may account, in part, for the apparent resistance. A situation of this sort has been described for pyknotic blood lymphocytes *in vivo.*[21]

It has been estimated that lymph node lymphocytes are about 11 times more sensitive *in vivo* than *in vitro* from the point of view of equivalent oxygen concentration.[28] The validity of such comparison is questionable, however,

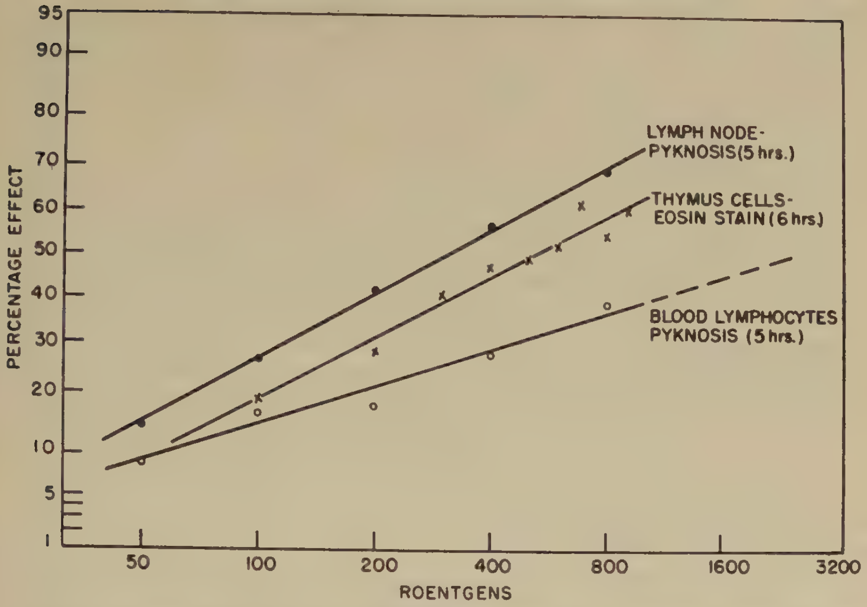

FIGURE 2. Comparative *in vitro* effects of X irradiation on lymphoid cells. (Lymph node and blood lymphocyte data from Trowell[21]; thymocyte data from Patt *et al.*[24])

when we recall that successful culturing of lymph nodes requires pure oxygen.[28, 29] Under these conditions, the average oxygen concentration exceeds that of nodes *in vivo* by a factor of 10. The greater oxygen requirement for lymphocyte survival *in vitro* points to differences in metabolism and in the accumulation of metabolic products. The comparatively small difference in sensitivity *in vivo* and *in vitro* when optimal culturing conditions are employed may be attributed, in part, to lactate accumulation in the cultures and, more generally, to differences in reaction time. The 22 hour ED_{50} for thymocytes *in vitro* is 114 r. which is consistent with the dosages required *in vivo.*

While the killing of lymphocytes appears to be mainly a direct consequence of their radiosensitivity, remote effects on lymphoid tissue have been observed from time to time. Price[30] has shown that a distant effect can be demonstrated, particularly when the volume of irradiated tissue is small. The relative magnitude of direct and remote effects on lymphoid tissue depends, for the most part, on the integral dose.

Temperature Effects

The effect of temperature on radiosensitivity has received considerable attention. There is no reason to believe, from a physical point of view, that the primary energy transfer resulting in ionization and excitation is temperature dependent. Extreme temperature changes may influence the response in certain instances, however, perhaps because of altered formation and diffusion of free radicals or of some alteration in configuration of the various biological targets. Other temperature dependencies during irradiation can generally be attributed to concomitant changes in cell division, in oxygen tension, or in blood flow, or to effects on recovery processes. When these considerations are not pertinent, it is generally observed that the immediate reaction of cells to ionizing radiation has a low temperature coefficient. This is the case with thymus lymphocytes, as shown by Schrek.[23]

The biological expression of the energy transfer, on the other hand, appears to be rate sensitive, the ultimate outcome depending on the respective temperature coefficients of the ensuing reactions of injury and of recovery for a given effect. This is illustrated in FIGURE 3, where it will be noted that the 50 per cent survival time of X-irradiated thymic cells is increased when the incubation temperature is decreased below 37° C.[23] The temperature coefficient for survival of nonirradiated cells is about three over the range of 17 to 42° C. Incubation temperatures in excess of 42° C. kill thymocytes before X-ray lethality can be manifest. Maximal protective effects appear when the

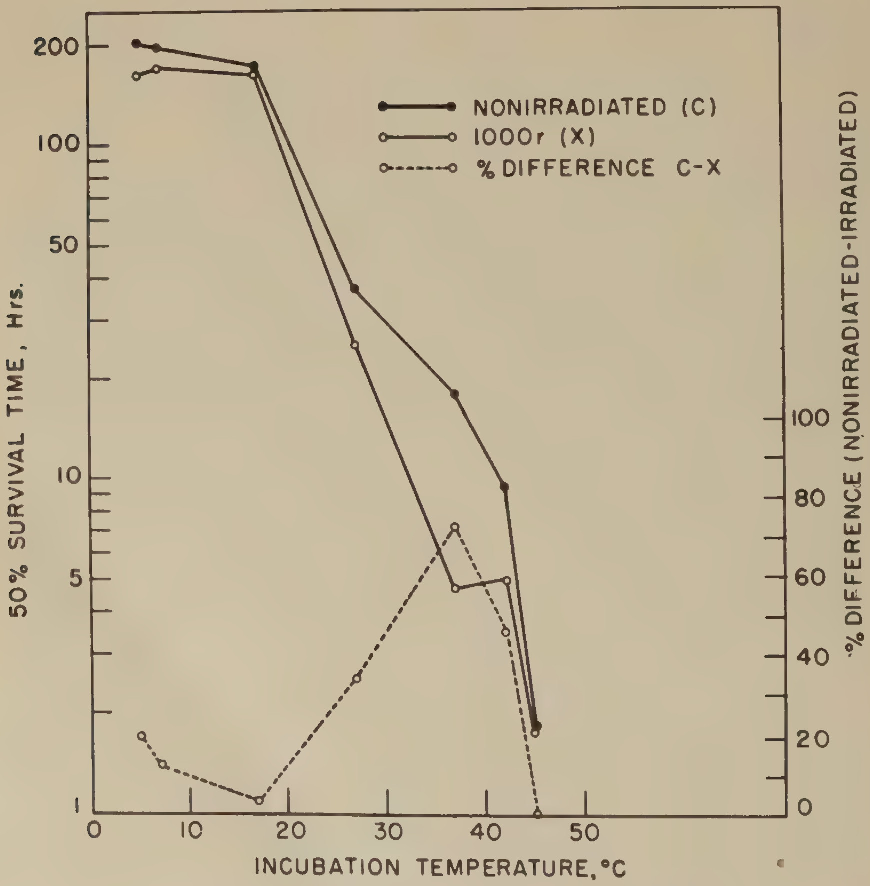

FIGURE 3. Survival time of thymus lymphocytes as a function of incubation temperature.[23]

temperature is about 17° C., and it may be recalled that an optimum of 18° C. has been observed by Stapleton *et al.*[31] for *Escherichia coli* in the presence of certain media. This similarity may be fortuitous, but it indicates, at any rate, that recovery can be influenced preferentially by appropriate temperature changes.

These results may be compared with our experiences with Amphibia, which showed that survival was greatly enhanced when frogs were kept at 6° C. continuously after X irradiation.[32] Survival was not influenced by altering the body temperature during irradiation. The protective effect of postirradiation cold was due apparently to a decrease in the rate of development of radiation damage rather than to any appreciable recovery, since removal of the animals from the cold after periods of 60 to 130 days resulted in a typical radiation picture. One wonders whether certain types of recovery, even in the frog, might not be influenced preferentially at intermediate temperatures. This question we are presently investigating.

Effects of pH

Changes in hydrogen ion concentration probably occur in the vicinity of an ionization track and may contribute to the development of radiation injury. The pH may also play a role in radiosensitivity by modifying the physicochemical characteristics of the various biological components or the rates of reaction subsequent to irradiation.

Marshak[33] has observed that the frequency of chromosome aberrations in plant cells is reduced when ammonium hydroxide is applied prior to X-ray exposure. The sensitivity of fern spores, *Drosophila* eggs, and *Paramecia* has also been shown by Zirkle[34-36] to depend on the pH during irradiation. The relationships are complex, with distinct sensitivity maxima and minima as the hydrogen ion concentration is increased or decreased. The effects do not correspond particularly to changes in the yield of radiochemical products with pH.[37] They may perhaps be related more to alterations in the behavior of the various protoplasmic colloids.

Significant pH effects have also been described by Schrek[23] for thymocytes, and by Trowell[28] for lymphocytes. These effects differ, however, from the effects described above since the changes are attributable to the pH after exposure to X rays. The pH dependency is remarkably similar in the case of thymus and lymph-node cells, even though the hydrogen ion concentration was changed after irradiation of the former and before irradiation of the latter. The results presented in FIGURE 4 reveal that sensitivity decreases sharply as the pH is diminished from 7.0 to 6.0. There is little change in sensitivity from pH 7.0 to about 8.5. Alteration in the pH of thymic cell suspensions from 6.6 to 7.8 during irradiation does not modify their survival. Nonirradiated cells will not survive incubation below pH 6.0. Survival is not altered by pH's of 6.0 to 8.5. The postirradiation effects of pH are apparently unrelated to autolytic phenomena, although other interpretations are not available. It may be remarked that the pH and temperature effects for thymus lymphocytes appear to differ with nitrogen mustard and X rays.[38] It is well known that there are many similarities in the action of these toxic agents.

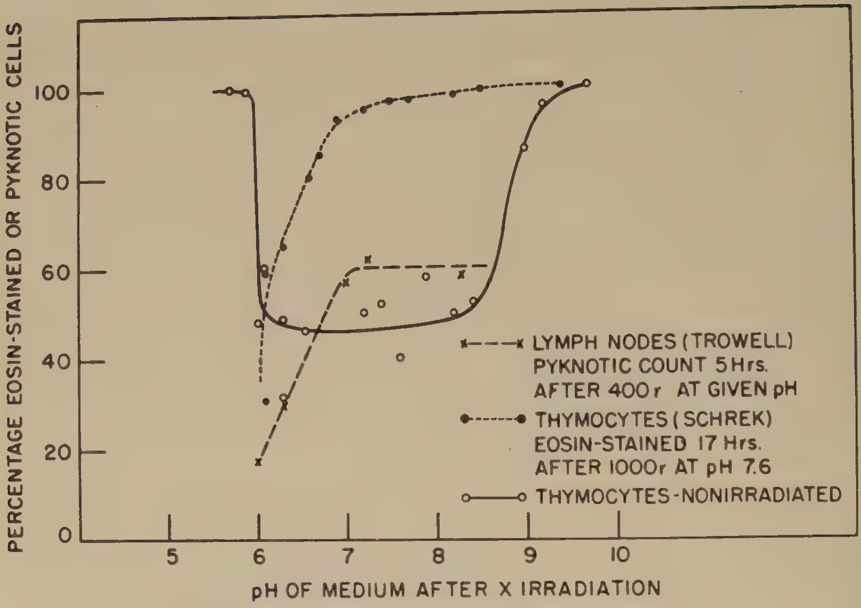

FIGURE 4. Effect of hydrogen ion concentration on radiosensitivity.[23, 28]

Effect of Oxygen

Certain forms of radiation sensitivity are known to be intimately related to oxygen tension, and it is appropriate to examine this relationship as it pertains to mammalian cells. Hypoxia during irradiation has been shown to reduce many of the effects of whole body or local exposure to gamma and X rays.[3] The pattern of the X-ray-oxygen dependence for rabbit thymus and rat-lymph-node lymphocytes and for mouse ascites tumor cells is presented in FIGURE 5. The effects observed with thymus cell and ascites tumor cell suspensions resemble closely those previously described for microorganisms, insects, and plants,[11] the sensitivity in air being about 2.5 to 3 times that in nitrogen. The oxygen effect, in general, increases sharply from 0 to 10 per cent oxygen and then plateaus at levels approaching the physiological or normal ambient tension. While this general picture also obtains for cultures of lymph-node lymphocytes,[28] the differences in sensitivity are considerably greater, and the range of the effect with oxygen tension is more extensive. Trowell[28] has suggested that the greater range may be an artifact. Since a rather steep oxygen gradient exists from the surface to the center of the lymph-node culture, the average oxygen tension in a system of this sort is considerably lower than the ambient tension.

These considerations are germane to many of the peculiarities in radiosensitivity of tumors and tumor fragments. Hall[12] has observed, for example, that the X-ray sensitivity of tumor fragments in air is an inverse function of their size, the implication being that the more centrally located cells are relatively anoxic, and that the anoxic area varies directly with the size of the

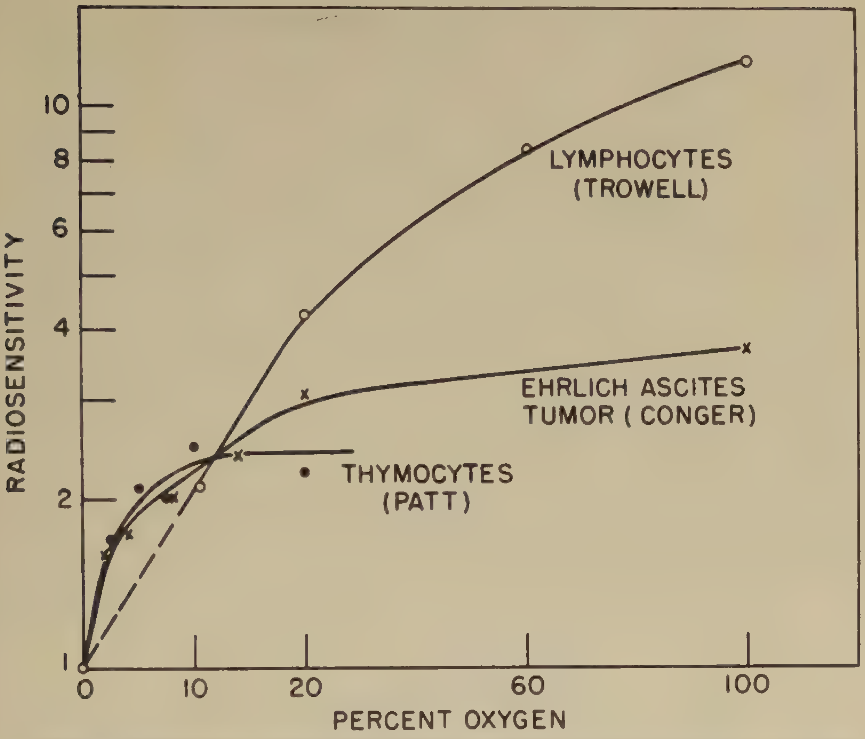

FIGURE 5. Radiosensitivity of lymph node and thymus lymphocytes and of ascites tumor cells as a function of oxygen tension.[11, 27, 28]

fragments. In further support of this observation, it was noted that the *in vivo* sensitivity of well-vascularized, rapidly growing tumors to local X irradiation is comparable to the *in vitro* sensitivity of tumor fragments irradiated in air when their oxygen consumption is depressed by cold or cyanide. The importance of oxygen tension in the response of certain solid tumors *in vivo* is obvious from the recent experiments by Gray *et al.*[11] Since it may be assumed that only a small oxygen gradient exists from the medium to individual cells in suspension, factors of oxygen consumption and diffusion would be of little consequence in cell suspensions. The fact that cyanide can potentiate X-ray sensitivity of various tissues, but not of cells in suspension, may be interpreted on this basis.[3]

The magnitude of the oxygen effect in rat lymphocytes, greater than in the other systems thus far investigated, is not readily explained, particularly in terms of the usual interpretation of effects of this sort.[3] Trowell[28] has speculated that chemical changes incidental to brief anoxia may play a greater role in the sensitivity of mammalian tissues than of plant or insect tissues, owing perhaps to the faster rate of metabolism of the former. Yet we may recall that suspensions of mouse ascites tumor cells exhibit a more or less typical oxygen-X ray sensitivity relationship.[11] For what it is worth, the sensitivity of rat-lymphocyte cultures is decreased somewhat by the addition of sodium lactate but not of glucose prior to X irradiation.[28] Intracellular accumulation of lactate in the lymph-node culture may perhaps complement other effects of anoxia, *e.g.*, on the yield of toxic breakdown products from cellular water. This would not appear to be sufficient, however, to account for the increased protective effect. It is of interest that sensitivity is not influenced appreciably when the cultures are irradiated in Tyrodes solution or in 0.65 per cent serum. Similar findings have been obtained by us with thymus lymphocyte suspensions, which suggests that the medium makes a negligible contribution to the toxic effect of X rays in these situations.

That the oxygen effect can not always be interpreted entirely in terms of immediate oxidative reactions in irradiated water is apparent also from other considerations. The data in FIGURE 6 reveal that protection does not occur if thymic-cell suspensions are chilled to 2° C. during equilibration with commercial nitrogen (*ca.* 0.1 per cent O_2) over a 15- or 30-minute period immediately prior to X irradiation. While this effect may perhaps represent an artifact, due to the difficulty in removing intracellular oxygen under these conditions, we have found that chilling, even for a brief period after irradiation, decreases significantly ($p < .01$) the protective effect of hypoxia. Other data emphasize

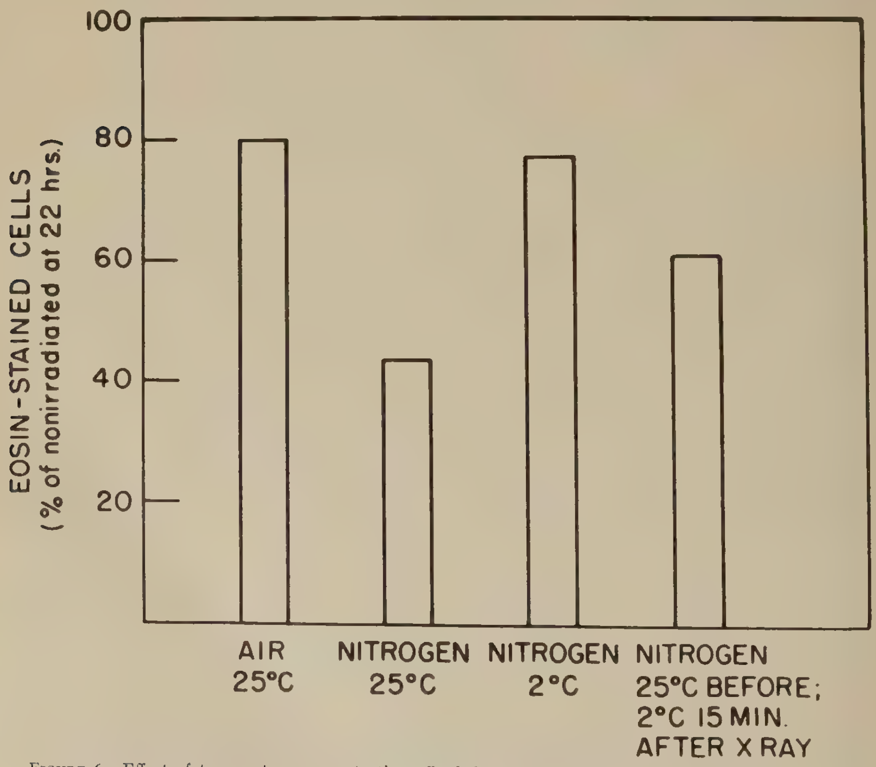

FIGURE 6. Effect of temperature on protection afforded X irradiated thymus lymphocytes by hypoxia.[46] (Nitrogen equilibration for 15 to 30 minutes at 2° or 25° C. before 200 r.)

the difficulties of interpretation. It has been observed, for example, that anoxic conditions after exposure to X rays exert almost as great a protective effect on the hematopoietic cells of tadpoles as similar treatment before and during the exposure.[39] Certain protective effects of prolonged anaerobiosis on suspensions of leukemic cells appear to be due mainly to the situation after irradiation.[22] There is the suggestion also that oxygen presence during X irradiation may affect the recovery of mitosis in grasshopper neuroblasts rather than its immediate depression.[22A] There is some uncertainty, however, concerning the dose-response relationships for mitotic block in this instance.

It is significant that oxygen plays a comparatively small part during irradiation of Ehrlich ascites tumor cells with fast neutrons in contrast to the marked effect with X rays.[11] An inverse relationship between the oxygen effect and ionization density has also been observed with *Vicia faba* roots[40] and *Tradescantia* microspores.[41] These facts parallel the greater oxygen dependence of radiochemical reactions resulting from the less densely ionizing radiations and provide a powerful argument that chemical intermediates derived from water, play a substantial role in the induction of radiation injuries. While we tacitly assume that oxygen modifies the nature of the chemical intermediates that are formed during exposure to gamma or X rays, alternative and complementary actions of oxygen must be involved in certain instances.

Protective Effect of Cysteine

There are numerous examples of chemical protection, and one is inclined generally to interpret them in radiochemical rather than in biological terms.[3] This interpretation implies, as in the case of anoxia, that the protective phenomena are due mainly to a decrease in the effectiveness of the radiation rather than to a basic change in the sensitivity of the biological system. We shall be concerned here with the effects of cysteine which, in certain respects at least, may be thought of as a characteristic example of protection by hydrogen donors.

The sensitivity of thymus lymphocytes is decreased by a factor of 2 to 3 when cysteine is added to the suspension prior to X irradiation (FIGURE 7). Similar protective effects occur with ascites tumor cells and with the whole animal. Protection of thymocytes depends upon cysteine concentration and time of administration but, significantly, is not a simple function of the sulfhydryl level during exposure.[24] Cysteine addition 15 to 30 minutes before irradiation is optimum, which suggests that some interaction is necessary. Unlike the picture with bacteria and animals, there is a definite, although lesser, protective effect when cysteine is added to the thymocyte suspension immediately after irradiation. This effect can be accounted for only to a slight extent by the persistence of toxic substances in the medium. The apparent discrepancy in the time course of protection of the various systems may be related perhaps to differences in the kinetics of reactions with cysteine and in the time constants for development of irreversible injury. It may be recalled that a postirradiation effect of cysteine has also been described for onion epidermis cells.[42]

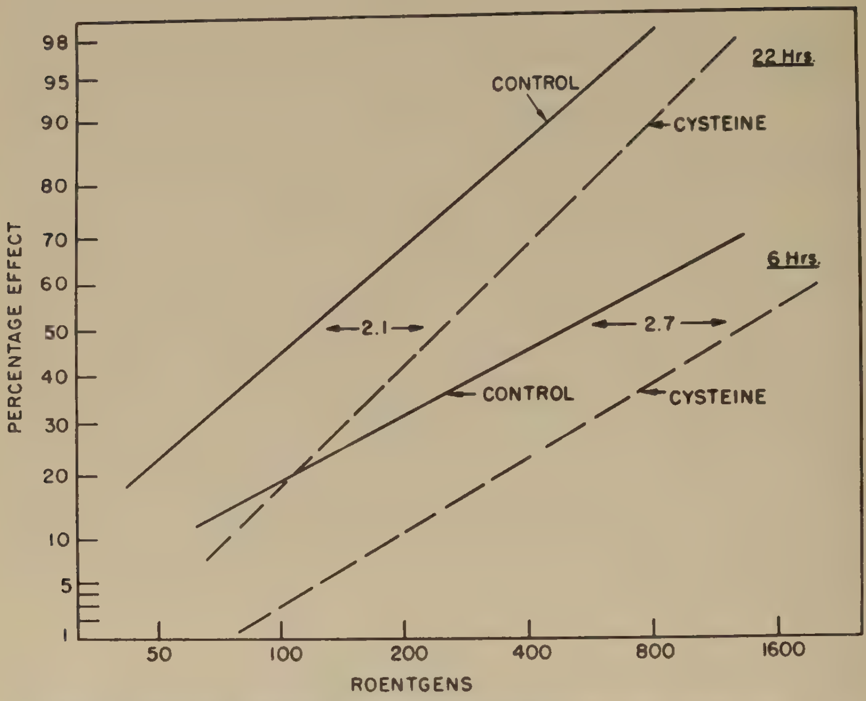

FIGURE 7. Dose-response relationship for control and cysteine protected thymus lymphocytes.[24, 27] (Cysteine, final concentration 0.02 M, added 15 minutes before X irradiation.)

In contrast to the finding that —SH compounds give some protection to bacteria in oxygen-free phosphate buffer,[43] it may be noted that cysteine does not protect thymic cells in the absence of oxygen. The resistance of packed thymocytes and the failure of cysteine to protect them may be attributed to their hypoxic state. Cells equilibrated with oxygen before packing by centrifugation appear to be as sensitive as cells in suspension, and they are readily protected by cysteine.[24] This is interpreted as evidence that cysteine acts at the level of the cell. A cellular site of action is implicated also from studies with tumor fragments.[44] It may be noted that there is a rapid uptake of oxygen when cysteine is added to the thymic cell suspension. There is also an increase in lactic acid.[45] These results suggest perhaps that cysteine action is related to the availability of intracellular oxygen.

Mechanisms other than the immediate oxidative reactions induced by activated water must be involved, however. It has been observed[46] that protection of thymocytes in suspension is dependent upon temperature during the first 30 to 60 minutes after irradiation (FIGURE 8). A brief period of chilling at 2° C. immediately after X-ray exposure completely reverses the protection. Temperature dependence of cysteine action before irradiation has also been shown. The latter may be thought of in terms of a decreased rate of reaction with oxygen in the cold. An explanation of the postirradiation temperature dependence for cysteine protection of thymus lymphocytes is not readily

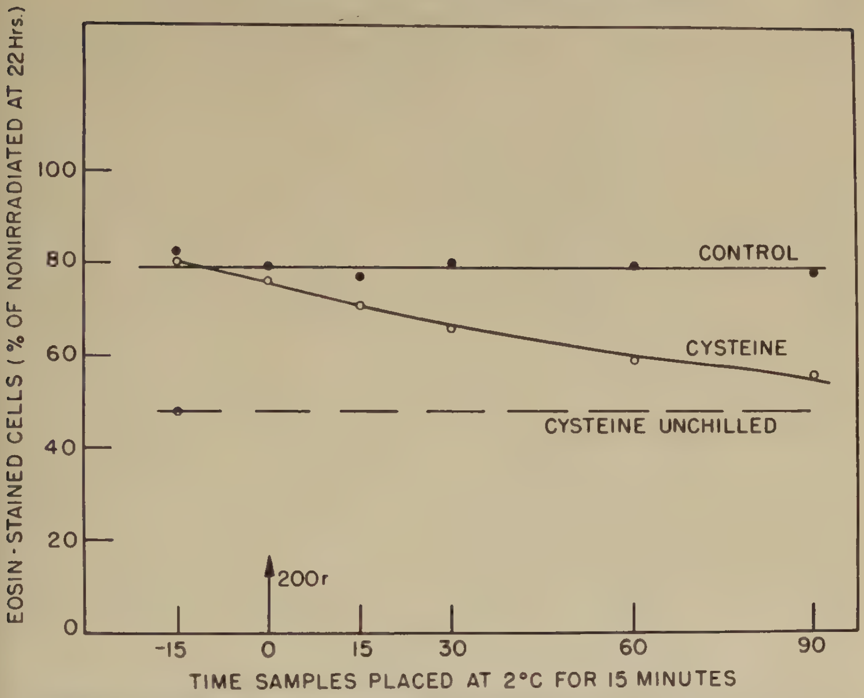

FIGURE 8. Effect of temperature on protection of X-irradiated thymus lymphocytes by cysteine.[46] (Cysteine, final concentration 0.02 M, added 15 minutes before X irradiation.)

apparent. It is clear, however, that we must inquire about the role of delayed and of chain reactions in the interpretation of these phenomena. The importance of considerations of this sort is indicated from recent work on the chemical aftereffects of ionizing radiations.[47] The pathways for reaction of organic radicals formed during irradiation may depend upon oxygen availability in addition to other factors. It is possible, for example, that decreased oxidation of cysteine at low temperature frees oxygen for reaction with persisting radicals to form organic peroxides. It is of interest that increased oxygen uptake was maintained for several hours with the cysteine concentration used, but that the critical postirradiation period for modification of the protection was about an hour. Cysteine protection is apparently unrelated to neutralization of hydrogen peroxide. In fact, cysteine potentiates or activates the effect of hydrogen peroxide on DNA solutions.[48] We may recall that a qualitatively similar, though lesser, temperature effect has been observed with hypoxia. Since the suspensions are not completely deoxygenated when commercial nitrogen is used, immediate postirradiation chilling may also increase intracellular oxygen above a critical level for reaction with chemical intermediates. The results with thymocytes may be contrasted with the chemical protective effects in bacteria.[49] Several modes of action apparently prevail in the protection of the various systems, owing perhaps to differences in the nature of the chemical intermediates and their reactions, the time required for develop-

TABLE 2

COMPARATIVE PROTECTION WITH CYSTEINE FOR SEVERAL EFFECTS ON MAMMALIAN SYSTEMS

Object	Effect	Per cent dose reduction	Reference
Thymocytes*	Eosin stainability	53	24
Ascites tumor*	Growth†	55	27
Solid tumor*	Transplantability†	18	44
Solid tumor*	Growth delay†	34	44
Lymphocytes†	Lymphopenia	31	26
Granulocytes†	Granulocytopenia	56	26
Spleen†	Involution	42	26
Mouse	Lethality—30 day	42	26

* *In vitro.*
† *In vivo.*

ment of irreversible injury and the contribution of the medium to the over-all effect.

In concluding, it is appropriate to refer to the pattern of cysteine protection for various effects in mammalian tissues. There is considerable evidence that the protection is a fairly general phenomenon, and that both destructive and regenerative processes can be modified.[3] This is apparent particularly when the effects can be quantitated in terms of radiation dose biological response parameters.[26] Uniformity of the protection is shown in TABLE 2. The comparatively low value obtained for transplantability of solid tumors may be related to the uncertainty concerning the dose-response curves. It is worth noting that *d* and *l* cysteine are equally effective in mice, but that all sulfhydryl substances are not protective to animals, perhaps because of differences in their biological fate. The action of cysteine and β mercaptoethylamine appears to be similar.[50] The latter is more efficient but is also more toxic than the former. Maximally tolerated doses of each give equivalent protection against acute radiation lethality. Suboptimal amounts seem to be completely additive.

Of considerable interest is the fact that cysteine protection against the lethal effects of fast neutron irradiation in mice is about half of that found with gamma irradiation.[52] This finding parallels the oxygen effect in irradiated systems and is supported by other data (TABLE 3). It may be remarked that the biological additivity of two briefly spaced X-ray doses in mice is a direct function of the cysteine dose preceding each exposure.[26] A similar additivity has been observed for the oxygen effect on broad bean roots.[51]

Uniformity of protection by cysteine suggests that the decisive action occurs at an early stage in the chain of events but does not necessarily imply protection against all radiation changes since different primary mechanisms may be involved in their development. For the present, these effects may be interpreted best in terms of the physicochemical ramifications of energy transfer in irradiated water. It would appear that protective chemicals of this sort may alter the effective radical concentration (*e.g.*, of OH and HO_2) either directly or indirectly by relative depletion of oxygen. The mechanisms may be more complex in certain instances and involve also effects on chain reactions initiated

TABLE 3

DEPENDENCE OF PROTECTIVE EFFECT OF ANOXIA OR CYSTEINE ON RADIATION QUALITY

Object	Radiation			Reference
	Alpha	Fast neutron	Gamma or X	
Vicia faba	+		++++	40
Tradescantia		++	++++	41
Allium*	+		++++	53
Ehrlich ascites tumor		+	++++	11
Mouse*		++	++++	52

* Refers to effect of cysteine.

by the primary interactions. The experimental findings, in general, are consistent with such assumptions, and there is little basis for postulating a more selective protection of one or another physiological mechanism.

Conclusions

There is no obvious common denominator to account for differences in radiosensitivity of the various mammalian cells and tissues. Although these differences can be related in certain instances to mitosis and growth, there are a number of exceptions to this condition. The over-all relationships are complex and are influenced by the interplay in the system as a whole.

The killing of lymphocytes appears to be mainly a direct consequence of their radiosensitivity. Sensitivity *in vivo* and *in vitro* is remarkably similar when extenuating factors are considered. The influence of environmental factors on sensitivity of lymphoid and other tissues is discussed.

The idea that radiation effects may be mediated by chemical vectors seems firmly established. Many of the protective effects of hypoxia and substances such as cysteine can be interpreted in terms of chemical intermediates derived from water and their reactions. It is clear, however, that we can not confine our thinking in this area.

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